(54) Title: GENETICALLY ENGINEERED VACCINE STRAIN

(57) Abstract

What is described is a modified vector, such as a recombinant poxvirus, particularly recombinant vaccinia virus, having enhanced safety. The modified recombinant virus has nonessential virus-encoded genetic functions inactivated therein so that virus has attenuated virulence. In one embodiment, the genetic functions are inactivated by deleting an open reading frame encoding a virulence factor. In another embodiment, the genetic functions are inactivated by insertional inactivation of an open reading frame encoding a virulence factor. What is also described is a vaccine containing the modified recombinant virus having nonessential virus-encoded genetic functions inactivated therein so that the vaccine has an increased level of safety compared to known recombinant virus vaccines.

GENETICALLY ENGINEERED VACCINE STRAIN

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Serial No. 07/713,967, filed June 11, 1991 which in turn is a continuation-in-part of application Serial No. 07/666,056, filed March 7, 1991, both of which are hereby incorporated herein by reference. Reference is also made to copending U.S. applications Serial Nos. 715,921, filed June 14, 1991, 736,254, filed July 26, 1991, 776,867, filed October 22, 1991, and 820,077, filed January 13, 1992, all of which are hereby incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to a modified poxvirus and to methods of making and using the same. More in particular, the invention relates to improved vectors for the insertion and expression of foreign genes for use as safe immunization vehicles to protect against a variety of pathogens.

Several publications are referenced in this application. Full citation to these references is found at the end of the specification immediately preceding the claims or where the publication is mentioned; and each of these publications is hereby incorporated herein by reference. These publications relate to the art to which this invention pertains.

BACKGROUND OF THE INVENTION

Vaccinia virus and more recently other poxviruses have been used for the insertion and expression of foreign genes. The basic technique of inserting foreign genes into live infectious poxvirus involves recombination between pox DNA sequences flanking a foreign genetic element in a donor plasmid and homologous sequences present in the rescuing poxvirus (Piccini et al., 1987).

Specifically, the recombinant poxviruses are constructed in two steps known in the art and analogous to the methods for creating synthetic recombinants of the vaccinia virus described in U.S. Patent Nos. 4,769,330, 4,772,848, and 4,603,112, and in copending application Serial No. 07/537,882, filed June 14, 1990, the disclosures of which are incorporated herein by reference. In this

regard reference is also made to copending U.S. application
Serial No. 537,890, filed June 14, 1990, also incorporated
herein by reference.

First, the DNA gene sequence to be inserted into the
virus, particularly an open reading frame from a non-pox
source, is placed into an *E. coli* plasmid construct into
which DNA homologous to a section of DNA of the poxvirus has
been inserted. Separately, the DNA gene sequence to be
inserted is ligated to a promoter. The promoter-gene
linkage is positioned in the plasmid construct so that the
promoter-gene linkage is flanked on both ends by DNA
homologous to a DNA sequence flanking a region of pox DNA
containing a nonessential locus. The resulting plasmid
construct is then amplified by growth within *E. coli*
bacteria (Clewell, 1972) and isolated (Clewell et al., 1969;
Maniatis et al., 1982).

Second, the isolated plasmid containing the DNA gene
sequence to be inserted is transfected into a cell culture,
e.g. chick embryo fibroblasts, along with the poxvirus.
Recombination between homologous pox DNA in the plasmid and
the viral genome respectively gives a poxvirus modified by
the presence, in a nonessential region of its genome, of
foreign DNA sequences. The term "foreign" DNA designates
exogenous DNA, particularly DNA from a non-pox source, that
codes for gene products not ordinarily produced by the
genome into which the exogenous DNA is placed.

Genetic recombination is in general the exchange of
homologous sections of DNA between two strands of DNA. In
certain viruses RNA may replace DNA. Homologous sections of
nucleic acid are sections of nucleic acid (DNA or RNA) which
have the same sequence of nucleotide bases.

Genetic recombination may take place naturally during
the replication or manufacture of new viral genomes within
the infected host cell. Thus, genetic recombination between
viral genes may occur during the viral replication cycle
that takes place in a host cell which is co-infected with
two or more different viruses or other genetic constructs.
A section of DNA from a first genome is used interchangeably
in constructing the section of the genome of a second co-

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infecting virus in which the DNA is homologous with that of
the first viral genome.

However, recombination can also take place between
sections of DNA in different genomes that are not perfectly
homologous. If one such section is from a first genome
homologous with a section of another genome except for the
presence within the first section of, for example, a genetic
marker or a gene coding for an antigenic determinant
inserted into a portion of the homologous DNA, recombination
can still take place and the products of that recombination
are then detectable by the presence of that genetic marker
or gene in the recombinant viral genome.

Successful expression of the inserted DNA genetic
sequence by the modified infectious virus requires two
conditions. First, the insertion must be into a
nonessential region of the virus in order that the modified
virus remain viable. The second condition for expression of
inserted DNA is the presence of a promoter in the proper
relationship to the inserted DNA. The promoter must be
placed so that it is located upstream from the DNA sequence
to be expressed.

Vaccinia virus has been used successfully to immunize
against smallpox, culminating in the worldwide eradication
of smallpox in 1980. In the course of its history, many
strains of vaccinia have arisen. These different strains
demonstrate varying immunogenicity and are implicated to
varying degrees with potential complications, the most
serious of which are post-vaccinial encephalitis and
generalized vaccinia (Behbehani, 1983).

With the eradication of smallpox, a new role for
vaccinia became important, that of a genetically engineered
vector for the expression of foreign genes. Genes encoding
a vast number of heterologous antigens have been expressed
in vaccinia, often resulting in protective immunity against
challenge by the corresponding pathogen (reviewed in
Tartaglia et al., 1990a).

The genetic background of the vaccinia vector has been
shown to affect the protective efficacy of the expressed
foreign immunogen. For example, expression of Epstein Barr

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Virus (EBV) gp340 in the Wyeth vaccine strain of vaccinia
virus did not protect cottontop tamarins against EBV virus
induced lymphoma, while expression of the same gene in the
WR laboratory strain of vaccinia virus was protective
(Morgan et al., 1988).

     A fine balance between the efficacy and the safety of a
vaccinia virus-based recombinant vaccine candidate is
extremely important. The recombinant virus must present the
immunogen(s) in a manner that elicits a protective immune
response in the vaccinated animal but lacks any significant
pathogenic properties. Therefore attenuation of the vector
strain would be a highly desirable advance over the current
state of technology.

     A number of vaccinia genes have been identified which
are non-essential for growth of the virus in tissue culture
and whose deletion or inactivation reduces virulence in a
variety of animal systems.

     The gene encoding the vaccinia virus thymidine kinase
(TK) has been mapped (Hruby et al., 1982) and sequenced
(Hruby et al., 1983; Weir et al., 1983). Inactivation or
complete deletion of the thymidine kinase gene does not
prevent growth of vaccinia virus in a wide variety of cells
in tissue culture. TK^- vaccinia virus is also capable of
replication *in vivo* at the site of inoculation in a variety
of hosts by a variety of routes.

     It has been shown for herpes simplex virus type 2 that
intravaginal inoculation of guinea pigs with TK^- virus
resulted in significantly lower virus titers in the spinal
cord than did inoculation with TK^+ virus (Stanberry et al.,
1985). It has been demonstrated that herpesvirus encoded TK
activity *in vitro* was not important for virus growth in
actively metabolizing cells, but was required for virus
growth in quiescent cells (Jamieson et al., 1974).

     Attenuation of TK^- vaccinia has been shown in mice
inoculated by the intracerebral and intraperitoneal routes
(Buller et al., 1985). Attenuation was observed both for
the WR neurovirulent laboratory strain and for the Wyeth
vaccine strain. In mice inoculated by the intradermal
route, TK^- recombinant vaccinia generated equivalent anti-

vaccinia neutralizing antibodies as compared with the
parental TK$^+$ vaccinia virus, indicating that in this test
system the loss of TK function does not significantly
decrease immunogenicity of the vaccinia virus vector.
Following intranasal inoculation of mice with TK$^-$ and TK$^+$
recombinant vaccinia virus (WR strain), significantly less
dissemination of virus to other locations, including the
brain, has been found (Taylor et al., 1991a).

     Another enzyme involved with nucleotide metabolism is
ribonucleotide reductase. Loss of virally encoded
ribonucleotide reductase activity in herpes simplex virus
(HSV) by deletion of the gene encoding the large subunit was
shown to have no effect on viral growth and DNA synthesis in
dividing cells *in vitro*, but severely compromised the
ability of the virus to grow on serum starved cells
(Goldstein et al., 1988). Using a mouse model for acute HSV
infection of the eye and reactivatable latent infection in
the trigeminal ganglia, reduced virulence was demonstrated
for HSV deleted of the large subunit of ribonucleotide
reductase, compared to the virulence exhibited by wild type
HSV (Jacobson et al., 1989).

     Both the small (Slabaugh et al., 1988) and large
(Schmitt et al., 1988) subunits of ribonucleotide reductase
have been identified in vaccinia virus. Insertional
inactivation of the large subunit of ribonucleotide
reductase in the WR strain of vaccinia virus leads to
attenuation of the virus as measured by intracranial
inoculation of mice (Child et al., 1990).

     The vaccinia virus hemagglutinin gene (HA) has been
mapped and sequenced (Shida, 1986). The HA gene of vaccinia
virus is nonessential for growth in tissue culture
(Ichihashi et al., 1971). Inactivation of the HA gene of
vaccinia virus results in reduced neurovirulence in rabbits
inoculated by the intracranial route and smaller lesions in
rabbits at the site of intradermal inoculation (Shida et
al., 1988). The HA locus was used for the insertion of
foreign genes in the WR strain (Shida et al., 1987),
derivatives of the Lister strain (Shida et al., 1988) and
the Copenhagen strain (Guo et al., 1989) of vaccinia virus.

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Recombinant HA⁻ vaccinia virus expressing foreign genes have
been shown to be immunogenic (Guo et al., 1989; Itamura et
al., 1990; Shida et al., 1988; Shida et al., 1987) and
protective against challenge by the relevant pathogen (Guo
et al., 1989; Shida et al., 1987).

     Cowpox virus (Brighton red strain) produces red
(hemorrhagic) pocks on the chorioallantoic membrane of
chicken eggs. Spontaneous deletions within the cowpox
genome generate mutants which produce white pocks (Pickup et
al., 1984). The hemorrhagic function (u) maps to a 38 kDa
protein encoded by an early gene (Pickup et al., 1986).
This gene, which has homology to serine protease inhibitors,
has been shown to inhibit the host inflammatory response to
cowpox virus (Palumbo et al., 1989) and is an inhibitor of
blood coagulation.

     The u gene is present in WR strain of vaccinia virus
(Kotwal et al., 1989b). Mice inoculated with a WR vaccinia
virus recombinant in which the u region has been inactivated
by insertion of a foreign gene produce higher antibody
levels to the foreign gene product compared to mice
inoculated with a similar recombinant vaccinia virus in
which the u gene is intact (Zhou et al., 1990). The u
region is present in a defective nonfunctional form in
Copenhagen strain of vaccinia virus (open reading frames B13
and B14 by the terminology reported in Goebel et al.,
1990a,b).

     Cowpox virus is localized in infected cells in
cytoplasmic A type inclusion bodies (ATI) (Kato et al.,
1959). The function of ATI is thought to be the protection
of cowpox virus virions during dissemination from animal to
animal (Bergoin et al., 1971). The ATI region of the cowpox
genome encodes a 160 kDa protein which forms the matrix of
the ATI bodies (Funahashi et al., 1988; Patel et al., 1987).
Vaccinia virus, though containing a homologous region in its
genome, generally does not produce ATI. In WR strain of
vaccinia, the ATI region of the genome is translated as a 94
kDa protein (Patel et al., 1988). In Copenhagen strain of
vaccinia virus, most of the DNA sequences corresponding to
the ATI region are deleted, with the remaining 3' end of the

region fused with sequences upstream from the ATI region to
form open reading frame (ORF) A26L (Goebel et al., 1990a,b).

A variety of spontaneous (Altenburger et al., 1989;
Drillien et al., 1981; Lai et al., 1989; Moss et al., 1981;
Paez et al., 1985; Panicali et al., 1981) and engineered
(Perkus et al., 1991; Perkus et al., 1989; Perkus et al.,
1986) deletions have been reported near the left end of the
vaccinia virus genome. A WR strain of vaccinia virus with a
10 kb spontaneous deletion (Moss et al., 1981; Panicali et
al., 1981) was shown to be attenuated by intracranial
inoculation in mice (Buller et al., 1985). This deletion
was later shown to include 17 potential ORFs (Kotwal et al.,
1988b). Specific genes within the deleted region include
the virokine N1L and a 35 kDa protein (C3L, by the
terminology reported in Goebel et al., 1990a,b).
Insertional inactivation of N1L reduces virulence by
intracranial inoculation for both normal and nude mice
(Kotwal et al., 1989a). The 35 kDa protein is secreted like
N1L into the medium of vaccinia virus infected cells. The
protein contains homology to the family of complement
control proteins, particularly the complement 4B binding
protein (C4bp) (Kotwal et al., 1988a). Like the cellular
C4bp, the vaccinia 35 kDa protein binds the fourth component
of complement and inhibits the classical complement cascade
(Kotwal et al., 1990). Thus the vaccinia 35 kDa protein
appears to be involved in aiding the virus in evading host
defense mechanisms.

The left end of the vaccinia genome includes two genes
which have been identified as host range genes, K1L (Gillard
et al., 1986) and C7L (Perkus et al., 1990). Deletion of
both of these genes reduces the ability of vaccinia virus to
grow on a variety of human cell lines (Perkus et al., 1990).

Fowlpox virus (FPV) is the prototypic virus of the
Avipox genus of the Poxvirus family. The virus causes an
economically important disease of poultry which has been
well controlled since the 1920's by the use of live
attenuated vaccines. Replication of the avipox viruses is
limited to avian species (Matthews, 1982b) and there are no
reports in the literature of the virus causing a productive

infection in any non-avian species including man. This host
restriction provides an inherent safety barrier to
transmission of the virus to other species and makes use of
FPV as a vaccine vector in poultry an attractive
proposition.

    FPV has been used advantageously as a vector expressing
antigens from poultry pathogens. The hemagglutinin protein
of a virulent avian influenza virus was expressed in an FPV
recombinant (Taylor et al., 1988a). After inoculation of
the recombinant into chickens and turkeys, an immune
response was induced which was protective against either a
homologous or a heterologous virulent influenza virus
challenge (Taylor et al., 1988a). FPV recombinants
expressing the surface glycoproteins of Newcastle Disease
Virus have also been developed (Taylor et al., 1990; Edbauer
et al., 1990).

    The use of live attenuated vectored vaccines present a
number of potential advantages. The vaccines are
inexpensive to produce and a number of poultry pathogens can
potentially be incorporated into one vector. The immunogen
is presented to the immune system in an authentic manner
such that both humoral and cell mediated responses can be
invoked. Because the disease agent is not replicating, side
effects of vaccination are minimal and the continual re-
introduction of the disease agent into the environment is
eliminated.

    It can be appreciated that provision of a novel vaccine
strains having enhanced safety would be a highly desirable
advance over the current state of technology. For instance,
so as to provide safer vaccines or safer products from the
expression of a gene or genes by a virus.

                    OBJECTS OF THE INVENTION

    It is therefore an object of this invention to provide
modified recombinant viruses, which viruses have enhanced
safety, and to provide a method of making such recombinant
viruses.

    It is an additional object of this invention to provide
a recombinant poxvirus vaccine having an increased level of
safety compared to known recombinant poxvirus vaccines.

It is a further object of this invention to provide a
modified vector for expressing a gene product in a host,
wherein the vector is modified so that it has attenuated
virulence in the host.

It is another object of this invention to provide a
method for expressing a gene product in a cell cultured *in
vitro* using a modified recombinant virus or modified vector
having an increased level of safety.

These and other objects and advantages of the present
invention will become more readily apparent after
consideration of the following.

STATEMENT OF THE INVENTION

In one aspect, the present invention relates to a
modified recombinant virus having inactivated virus-encoded
genetic functions so that the recombinant virus has
attenuated virulence and enhanced safety. The functions can
be non-essential, or associated with virulence. The virus
is advantageously a poxvirus, particularly a vaccinia virus
or an avipox virus, such as fowlpox virus and canarypox
virus.

In another aspect, the present invention relates to a
vaccine for inducing an immunological response in a host
animal inoculated with the vaccine, said vaccine including a
carrier and a modified recombinant virus having inactivated
nonessential virus-encoded genetic functions so that the
recombinant virus has attenuated virulence and enhanced
safety. The virus used in the vaccine according to the
present invention is advantageously a poxvirus, particularly
a vaccinia virus or an avipox virus, such as fowlpox virus
and canarypox virus.

In yet another aspect, the present invention relates to
an immunogenic composition containing a modified recombinant
virus having inactivated nonessential virus-encoded genetic
functions so that the recombinant virus has attenuated
virulence and enhanced safety. The modified recombinant
virus includes, within a non-essential region of the virus
genome, a heterologous DNA sequence which encodes an
antigenic protein derived from a pathogen wherein the
composition, when administered to a host, is capable of

inducing an immunological response specific to the protein
encoded by the pathogen.

In a further aspect, the present invention relates to a
method for expressing a gene product in a cell cultured *in
vitro* by introducing into the cell a modified recombinant
virus having attenuated virulence and enhanced safety.

In a still further aspect, the present invention
relates to a modified recombinant virus having nonessential
virus-encoded genetic functions inactivated therein so that
the virus has attenuated virulence, and wherein the modified
recombinant virus further contains DNA from a heterologous
source in a nonessential region of the virus genome. In
particular, the genetic functions are inactivated by
deleting an open reading frame encoding a virulence factor
or by utilizing naturally host restricted viruses. The virus
used according to the present invention is advantageously a
poxvirus, particularly a vaccinia virus or an avipox virus,
such as fowlpox virus and canarypox virus. Advantageously,
the open reading frame is selected from the group consisting
of J2R, B13R + B14R, A26L, A56R, C7L - K1L, and I4L (by the
terminology reported in Goebel et al., 1990a,b). In this
respect, the open reading frame comprises a thymidine kinase
gene, a hemorrhagic region, an A type inclusion body region,
a hemagglutinin gene, a host range gene region or a large
subunit, ribonucleotide reductase.

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description, given by way of
example, but not intended to limit the invention solely to
the specific embodiments described, may best be understood
in conjunction with the accompanying drawings, in which:

FIG. 1 schematically shows a method for the
construction of plasmid pSD460 for deletion of thymidine
kinase gene and generation of recombinant vaccinia virus
vP410;

FIG. 2 schematically shows a method for the
construction of plasmid pSD486 for deletion of hemorrhagic
region and generation of recombinant vaccinia virus vP553;

FIG. 3 schematically shows a method for the construction of plasmid pMP494Δ for deletion of ATI region and generation of recombinant vaccinia virus vP618;

FIG. 4 schematically shows a method for the construction of plasmid pSD467 for deletion of hemagglutinin gene and generation of recombinant vaccinia virus vP723;

FIG. 5 schematically shows a method for the construction of plasmid pMPCSK1Δ for deletion of gene cluster [C7L - K1L] and generation of recombinant vaccinia virus vP804;

FIG. 6 schematically shows a method for the construction of plasmid pSD548 for deletion of large subunit, ribonucleotide reductase and generation of recombinant vaccinia virus vP866 (NYVAC);

FIG. 7 schematically shows a method for the construction of plasmid pRW842 for insertion of rabies glycoprotein G gene into the TK deletion locus and generation of recombinant vaccinia virus vP879;

FIG. 8 is a map of the EBV coding regions inserted into EBV Triple.1 plasmid;

FIG. 9 shows the DNA sequence (SEQ ID NO:213) of the synthetic spsAg gene and modified synthetic vaccinia virus H6 early/late promoter with the predicted amino acid sequence (SEQ ID NO:214);

FIG. 10 schematically shows a method for the construction of recombinant vaccinia virus vP856;

FIG. 11 shows the DNA sequence (SEQ ID NO:215) of the u promoter/lpsAg gene with the predicted amino acid sequence (SEQ ID NO:216);

FIG. 12 schematically shows a method for the construction of recombinant vaccinia virus vP896;

FIG. 13 shows the DNA sequence (SEQ ID NO:87) of the I3L promoter/S12/core gene with the predicted amino acid sequence (SEQ ID NO:217);

FIG. 14 schematically shows a method for the construction of recombinant vaccinia virus vP919;

FIG. 15 shows the DNA sequence (SEQ ID NO:218) of the EPV 42 kDa promoter/lpsAg gene with the predicted amino acid sequence (SEQ ID NO:219);

FIG. 16 shows the DNA sequence (SEQ ID NO:217) of a canarypox PvuII fragment containing the C5 ORF.

FIG. 17 schematically shows a method for the construction of recombinant canarypox virus vCP65 (ALVAC-RG);

FIG. 18 is a schematic of the JEV coding regions inserted in the vaccinia viruses vP555, vP825, vP908, vP923, vP857 and vP864;

FIG. 19 is a schematic of the YF coding regions inserted in the vaccinia viruses vP766, vP764, vP869, vP729 and vP725;

FIG 20 is a schematic of the DEN coding regions inserted in the vaccinia viruses vP867, vP962 and vP955;

FIG. 21 shows the nucleotide sequence (SEQ ID NO:221) of a 3661 base pair fragment of TROVAC DNA containing the F8 ORF;

FIG. 22 shows the DNA sequence (SEQ ID NO:222) of a 2356 base pair fragment of TROVAC DNA containing the F7 ORF;

FIG. 23 shows the nucleotide sequence of EIV HA (A1/Prague/56) (SEQ ID NO:279);

FIG. 24 shows the nucleotide sequence of EIV HA (A2/Fontainebleu/79) (SEQ ID NO:284);

FIG. 25 shows the nucleotide sequence of EIV HA (A2/Suffolk/89) (SEQ ID NO:300);

FIG. 26 shows the nucleotide sequence of FeLV-B Envelope Gene (SEQ ID NO:310);

FIG. 27 shows the nucleotide sequence of FeLV-A gag and partial pol genes (SEQ ID NO:324);

FIG. 28 shows the nucleotide sequence of the FHV-1 CO strain gD homolog gene (SEQ ID NO:290);

FIG. 29 shows the consensus F nucleotide sequence (mumps) represented by pURF3 (SEQ ID NO:370);

FIG. 30 shows the consensus HN nucleotide sequence (mumps) represented by pURHN5 (SEQ ID NO:371);

FIG. 31 shows the cytotoxic responses of spleen cells of mice and immunized with vaccinia virus or canarypox virus

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vectors (NYVAC, ALVAC) or with vaccinia virus or canarypox
virus recombinants expressing HIV III B env (vP911, vCP112);

FIG. 32 shows the sensitivity of the cytotoxic effector
cells from the spleens of mice immunized with vCP112 to
antibodies against cytotoxic T lymphocyte cell surface
antigens Thy 1.2 and Lyt 2.2;

FIG. 33 shows the specificity of cytotoxic T lymphocyte
antigen receptor for the HIV III B hypervariable V3 loop of
gp120, but not for the V3 loop of HIV MN or SF2;

FIG. 34 shows the antibody responses to HIV III B gp120
of mice immunized with vectors (NYVAC, ALVAC) or with
vaccinia virus recombinant vP911 or canarypox recombinant
vCP112 expressing HIV-1 env (inverted triangle indicates
time of administration of second inoculation);

FIG. 35 shows graph of rabies neutralizing antibody
titers (RFFIT, IU/ml), booster effect of HDC and vCP65
($10^{5.5}$ TCID50) in volunteers previously immunized with
either the same or the alternate vaccine (vaccines given at
days 0, 28 and 180, antibody titers measured at days 0, 7,
28, 35, 56, 173, 187 and 208);

FIG. 36 shows JEV cDNA sequences contained in vP908,
vP555, vP923 and vP829;

FIG. 37 shows NEUT and HAI activities observed in swine
immunized on days 0 and 28 with vP908, vP923, vP866 and PBS
(arrows indicated days of inoculation);

FIG. 38 shows time course of viremia detected in
individual pigs of each group immunized with PBS, vP866,
vP908 or vP923 and then challenged with the B-2358/84 strain
of JEV;

Fig 39 shows schematically the ORFs deleted to generate
NYVAC;

DETAILED DESCRIPTION OF THE INVENTION

To develop a new vaccinia vaccine strain, NYVAC
(vP866), the Copenhagen vaccine strain of vaccinia virus was
modified by the deletion of six nonessential regions of the
genome encoding known or potential virulence factors. The
sequential deletions are detailed below. All designations
of vaccinia restriction fragments, open reading frames and

nucleotide positions are based on the terminology reported
in Goebel et al., 1990a,b.

The deletion loci were also engineered as recipient
loci for the insertion of foreign genes.

The regions deleted in NYVAC are listed below. Also
listed are the abbreviations and open reading frame
designations for the deleted regions (Goebel et al.,
1990a,b) and the designation of the vaccinia recombinant
(vP) containing all deletions through the deletion
specified:

     (1) thymidine kinase gene (TK; J2R) vP410;

     (2) hemorrhagic region (u; B13R + B14R) vP553;

     (3) A type inclusion body region (ATI; A26L) vP618;

     (4) hemagglutinin gene (HA; A56R) vP723;

     (5) host range gene region (C7L - K1L) vP804; and

     (6) large subunit, ribonucleotide reductase (I4L) vP866
         (NYVAC).

     DNA Cloning and Synthesis. Plasmids were constructed,
screened and grown by standard procedures (Maniatis et al.,
1982; Perkus et al., 1985; Piccini et al., 1987).
Restriction endonucleases were obtained from Bethesda
Research Laboratories, Gaithersburg, MD, New England
Biolabs, Beverly, MA; and Boehringer Mannheim Biochemicals,
Indianapolis, IN. Klenow fragment of E. coli polymerase was
obtained from Boehringer Mannheim Biochemicals. BAL-31
exonuclease and phage T4 DNA ligase were obtained from New
England Biolabs. The reagents were used as specified by the
various suppliers.

     Synthetic oligodeoxyribonucleotides were prepared on a
Biosearch 8750 or Applied Biosystems 380B DNA synthesizer as
previously described (Perkus et al., 1989). DNA sequencing
was performed by the dideoxy-chain termination method
(Sanger et al., 1977) using Sequenase (Tabor et al., 1987)
as previously described (Guo et al., 1989). DNA
amplification by polymerase chain reaction (PCR) for
sequence verification (Engelke et al., 1988) was performed
using custom synthesized oligonucleotide primers and GeneAmp
DNA amplification Reagent Kit (Perkin Elmer Cetus, Norwalk,
CT) in an automated Perkin Elmer Cetus DNA Thermal Cycler.

Excess DNA sequences were deleted from plasmids by
restriction endonuclease digestion followed by limited
digestion by BAL-31 exonuclease and mutagenesis (Mandecki,
1986) using synthetic oligonucleotides.

     Cells, Virus, and Transfection. The origins and
conditions of cultivation of the Copenhagen strain of
vaccinia virus has been previously described (Guo et al.,
1989). Generation of recombinant virus by recombination, *in
situ* hybridization of nitrocellulose filters and screening
for B-galactosidase activity are as previously described
(Panicali et al., 1982; Perkus et al., 1989).

     A better understanding of the present invention and of
its many advantages will be had from the following examples,
given by way of illustration.

Example 1 - **CONSTRUCTION OF PLASMID pSD460 FOR
               DELETION OF THYMIDINE KINASE GENE (J2R)**

     Referring now to FIG. 1, plasmid pSD406 contains
vaccinia HindIII J (pos. 83359 - 88377) cloned into pUC8.
pSD406 was cut with HindIII and PvuII, and the 1.7 kb
fragment from the left side of HindIII J cloned into pUC8
cut with HindIII/SmaI, forming pSD447. pSD447 contains the
entire gene for J2R (pos. 83855 - 84385). The initiation
codon is contained within an NlaIII site and the termination
codon is contained within an SspI site. Direction of
transcription is indicated by an arrow in FIG. 1.

     To obtain a left flanking arm, a 0.8 kb HindIII/EcoRI
fragment was isolated from pSD447, then digested with NlaIII
and a 0.5 kb HindIII/NlaIII fragment isolated. Annealed
synthetic oligonucleotides MPSYN43/MPSYN44 (SEQ ID NO:1/SEQ
ID NO:2)

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                                 SmaI
MPSYN43  5'     TAATTAACTAGCTACCCGGG      3'
MPSYN44  3' GTACATTAATTGATCGATGGGCCCTTAA  5'
             NlaIII                 EcoRI
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were ligated with the 0.5 kb HindIII/NlaIII fragment into
pUC18 vector plasmid cut with HindIII/EcoRI, generating
plasmid pSD449.

     To obtain a restriction fragment containing a vaccinia
right flanking arm and pUC vector sequences, pSD447 was cut
with SspI (partial) within vaccinia sequences and HindIII at

the pUC/vaccinia junction, and a 2.9 kb vector fragment
isolated. This vector fragment was ligated with annealed
synthetic oligonucleotides MPSYN45/MPSYN46 (SEQ ID NO:3/SEQ
ID NO:4)

```
          HindIII SmaI
MPSYN45 5' AGCTTCCCGGGTAAGTAATACGTCAAGGAGAAAACGAA
MPSYN46 3'      AGGGCCCATTCATTATGCAGTTCCTCTTTTGCTT

             NotI          SspI
       ACGATCTGTAGTTAGCGGCCGCCTAATTAACTAAT 3' MPSYN45
       TGCTAGACATCAATCGCCGGCGGATTAATTGATTA 5' MPSYN46
```

generating pSD459.

     To combine the left and right flanking arms into one
plasmid, a 0.5 kb HindIII/SmaI fragment was isolated from
pSD449 and ligated with pSD459 vector plasmid cut with
HindIII/SmaI, generating plasmid pSD460. pSD460 was used as
donor plasmid for recombination with wild type parental
vaccinia virus Copenhagen strain VC-2. ^{32}P labelled probe
was synthesized by primer extension using MPSYN45 (SEQ ID
NO:3) as template and the complementary 20mer
oligonucleotide MPSYN47 (SEQ ID NO:5)
(5' TTAGTTAATTAGGCGGCCGC 3') as primer. Recombinant virus
vP410 was identified by plaque hybridization.

**Example 2 — CONSTRUCTION OF PLASMID pSD486 FOR
              DELETION OF HEMORRHAGIC REGION (B13R + B14R)**

     Referring now to FIG. 2, plasmid pSD419 contains
vaccinia SalI G (pos. 160,744-173,351) cloned into pUC8.
pSD422 contains the contiguous vaccinia SalI fragment to the
right, SalI J (pos. 173,351-182,746) cloned into pUC8. To
construct a plasmid deleted for the hemorrhagic region, u,
B13R – B14R (pos. 172,549 – 173,552), pSD419 was used as the
source for the left flanking arm and pSD422 was used as the
source of the right flanking arm. The direction of
transcription for the u region is indicated by an arrow in
FIG. 2.

     To remove unwanted sequences from pSD419, sequences to
the left of the NcoI site (pos. 172,253) were removed by
digestion of pSD419 with NcoI/SmaI followed by blunt ending
with Klenow fragment of E. coli polymerase and ligation
generating plasmid pSD476. A vaccinia right flanking arm
was obtained by digestion of pSD422 with HpaI at the

termination codon of B14R and by digestion with <u>Nru</u>I 0.3 kb
to the right. This 0.3 kb fragment was isolated and ligated
with a 3.4 kb <u>Hinc</u>II vector fragment isolated from pSD476,
generating plasmid pSD477. The location of the partial
deletion of the vaccinia <u>u</u> region in pSD477 is indicated by
a triangle. The remaining B13R coding sequences in pSD477
were removed by digestion with <u>Cla</u>I/<u>Hpa</u>I, and the resulting
vector fragment was ligated with annealed synthetic
oligonucleotides SD22mer/SD20mer (SEQ ID NO:6/SEQ ID NO:7)

```
              ClaI           BamHI HpaI
SD22mer  5'  CGATTACTATGAAGGATCCGTT   3'
SD20mer  3'    TAATGATACTTCCTAGGCAA   5'
```

generating pSD479. pSD479 contains an initiation codon
(underlined) followed by a <u>Bam</u>HI site. To place E. coli
Beta-galactosidase in the B13-B14 (<u>u</u>) deletion locus under
the control of the <u>u</u> promoter, a 2 kb <u>Bam</u>HI fragment
containing the Beta-galactosidase gene (Shapira et al.,
1983) was inserted into the <u>Bam</u>HI site of pSD479, generating
pSD479BG. pSD479BG was used as donor plasmid for
recombination with vaccinia virus vP410. Recombinant
vaccinia virus vP533 was isolated as a blue plaque in the
presence of chromogenic substrate X-gal. In vP533 the B13R-
B14R region is deleted and is replaced by Beta-
galactosidase.

      To remove Beta-galactosidase sequences from vP533,
plasmid pSD486, a derivative of pSD477 containing a
polylinker region but no initiation codon at the <u>u</u> deletion
junction, was utilized. First the <u>Cla</u>I/<u>Hpa</u>I vector fragment
from pSD477 referred to above was ligated with annealed
synthetic oligonucleotides SD42mer/SD40mer (SEQ ID NO:8/SEQ
ID NO:9)

```
              ClaI            SacI        XhoI        HpaI
SD42mer  5'  CGATTACTAGATCTGAGCTCCCCGGGCTCGAGGGATCCGTT   3'
SD40mer  3'    TAATGATCTAGACTCGAGGGGCCCGAGCTCCCTAGGCAA   5'
                   BglII        SmaI         BamHI
```

generating plasmid pSD478. Next the <u>Eco</u>RI site at the
pUC/vaccinia junction was destroyed by digestion of pSD478
with <u>Eco</u>RI followed by blunt ending with Klenow fragment of
E. coli polymerase and ligation, generating plasmid

pSD478E⁻. pSD478E⁻ was digested with <u>Bam</u>HI and <u>Hpa</u>I and
ligated with annealed synthetic oligonucleotides HEM5/HEM6
(SEQ ID NO:10/SEQ ID NO:11)

```
         BamHI EcoRI   HpaI
HEM5  5'  GATCCGAATTCTAGCT  3'
HEM6  3'      GCTTAAGATCGA  5'
```

generating plasmid pSD486. pSD486 was used as donor plasmid
for recombination with recombinant vaccinia virus vP533,
generating vP553, which was isolated as a clear plaque in
the presence of X-gal.

**Example 3 - CONSTRUCTION OF PLASMID pMP494Δ
                FOR DELETION OF ATI REGION (A26L)**

     Referring now to FIG. 3, pSD414 contains <u>Sal</u>I B cloned
into pUC8. To remove unwanted DNA sequences to the left of
the A26L region, pSD414 was cut with <u>Xba</u>I within vaccinia
sequences (pos. 137,079) and with <u>Hind</u>III at the
pUC/vaccinia junction, then blunt ended with Klenow fragment
of *E. coli* polymerase and ligated, resulting in plasmid
pSD483. To remove unwanted vaccinia DNA sequences to the
right of the A26L region, pSD483 was cut with <u>Eco</u>RI (pos.
140,665 and at the pUC/vaccinia junction) and ligated,
forming plasmid pSD484. To remove the A26L coding region,
pSD484 was cut with <u>Nde</u>I (partial) slightly upstream from
the A26L ORF (pos. 139,004) and with <u>Hpa</u>I (pos. 139,889)
slightly downstream from the A26L ORF. The 5.2 kb vector
fragment was isolated and ligated with annealed synthetic
oligonucleotides ATI3/ATI4 (SEQ ID NO:12/SEQ ID NO:13)

```
       NdeI
ATI3  5' TATGAGTAACTTAACTCTTTTGTTAATTAAAAGTATATTCAAAAAATAAGT
ATI4  3'   ACTCATTGAATTGAGAAAACAATTAATTTTCATATAAGTTTTTTATTCA
```

```
              BglII EcoRI HpaI
       TATATAAATAGATCTGAATTCGTT  3' ATI3
       ATATATTTATCTAGACTTAAGCAA  5' ATI4
```

reconstructing the region upstream from A26L and replacing
the A26L ORF with a short polylinker region containing the
restriction sites <u>Bgl</u>II, <u>Eco</u>RI and <u>Hpa</u>I, as indicated above.
The resulting plasmid was designated pSD485. Since the
<u>Bgl</u>II and <u>Eco</u>RI sites in the polylinker region of pSD485 are
not unique, unwanted <u>Bgl</u>II and <u>Eco</u>RI sites were removed from
plasmid pSD483 (described above) by digestion with <u>Bgl</u>II

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(pos. 140,136) and with EcoRI at the pUC/vaccinia junction,
followed by blunt ending with Klenow fragment of *E. coli*
polymerase and ligation. The resulting plasmid was
designated pSD489. The 1.8 kb ClaI (pos. 137,198)/EcoRV
(pos. 139,048) fragment from pSD489 containing the A26L ORF
was replaced with the corresponding 0.7 kb polylinker-
containing ClaI/EcoRV fragment from pSD485, generating
pSD492. The BglII and EcoRI sites in the polylinker region
of pSD492 are unique.

A 3.3 kb BglII cassette containing the *E. coli* Beta-
galactosidase gene (Shapira et al., 1983) under the control
of the vaccinia 11 kDa promoter (Bertholet et al., 1985;
Perkus et al., 1990) was inserted into the BglII site of
pSD492, forming pSD493KBG. Plasmid pSD493KBG was used in
recombination with rescuing virus vP553. Recombinant
vaccinia virus, vP581, containing Beta-galactosidase in the
A26L deletion region, was isolated as a blue plaque in the
presence of X-gal.

To generate a plasmid for the removal of Beta-
galactosidase sequences from vaccinia recombinant virus
vP581, the polylinker region of plasmid pSD492 was deleted
by mutagenesis (Mandecki, 1986) using synthetic
oligonucleotide MPSYN177 (SEQ ID NO:14)
(5' AAAATGGGCGTGGATTGTTAACTTTATATAACTTATTTTTTGAATATAC 3').
In the resulting plasmid, pMP494Δ, vaccinia DNA encompassing
positions [137,889 – 138,937], including the entire A26L ORF
is deleted. Recombination between the pMP494Δ and the Beta-
galactosidase containing vaccinia recombinant, vP581,
resulted in vaccinia deletion mutant vP618, which was
isolated as a clear plaque in the presence of X-gal.

**Example 4 – CONSTRUCTION OF PLASMID pSD467 FOR
              DELETION OF HEMAGGLUTININ GENE (A56R)**

Referring now to FIG. 4, vaccinia SalI G restriction
fragment (pos. 160,744-173,351) crosses the HindIII A/B
junction (pos. 162,539). pSD419 contains vaccinia SalI G
cloned into pUC8. The direction of transcription for the
hemagglutinin (HA) gene is indicated by an arrow in FIG. 4.
Vaccinia sequences derived from HindIII B were removed by
digestion of pSD419 with HindIII within vaccinia sequences

and at the pUC/vaccinia junction followed by ligation. The
resulting plasmid, pSD456, contains the HA gene, A56R,
flanked by 0.4 kb of vaccinia sequences to the left and 0.4
kb of vaccinia sequences to the right. A56R coding
sequences were removed by cutting pSD456 with RsaI (partial;
pos. 161,090) upstream from A56R coding sequences, and with
EagI (pos. 162,054) near the end of the gene. The 3.6 kb
RsaI/EagI vector fragment from pSD456 was isolated and
ligated with annealed synthetic oligonucleotides MPSYN59
(SEQ ID NO:15), MPSYN62 (SEQ ID NO:16), MPSYN60 (SEQ ID
NO:17), and MPSYN 61
(SEQ ID NO:18)

```
              RsaI
MPSYN59 5' ACACGAATGATTTTCTAAAGTATTTGGAAAGTTTTATAGGT-
MPSYN62 3' TGTGCTTACTAAAAGATTTCATAAACCTTTCAAAATATCCA-

MPSYN59    AGTTGATAGAACAAAATACATAATTT 3'
MPSYN62    TCAACTATCT 5'

MPSYN60 5'                   TGTAAAAATAAATCACTTTTTATA-
MPSYN61 3' TGTTTTATGTATTAAAACATTTTTATTTAGTGAAAAATAT-

           BglII  SmaI  PstI  EagI
MPSYN60    CTAAGATCTCCCGGGCTGCAGC      3'
MPSYN61    GATTCTAGAGGGCCCGACGTCGCCGG  5'
```

reconstructing the DNA sequences upstream from the A56R ORF
and replacing the A56R ORF with a polylinker region as
indicated above. The resulting plasmid is pSD466. The
vaccinia deletion in pSD466 encompasses positions [161,185-
162,053]. The site of the deletion in pSD466 is indicated
by a triangle in FIG. 4.

     A 3.2 kb BglII/BamHI (partial) cassette containing the
E. coli Beta-galactosidase gene (Shapira et al., 1983) under
the control of the vaccinia 11 kDa promoter (Bertholet et
al., 1985; Guo et al., 1989) was inserted into the BglII
site of pSD466, forming pSD466KBG. Plasmid pSD466KBG was
used in recombination with rescuing virus vP618.
Recombinant vaccinia virus, vP708, containing Beta-
galactosidase in the A56R deletion, was isolated as a blue
plaque in the presence of X-gal.

     Beta-galactosidase sequences were deleted from vP708
using donor plasmid pSD467. pSD467 is identical to pSD466,
except that EcoRI, SmaI and BamHI sites were removed from

the pUC/vaccinia junction by digestion of pSD466 with
EcoRI/BamHI followed by blunt ending with Klenow fragment of
E. coli polymerase and ligation. Recombination between
vP708 and pSD467 resulted in recombinant vaccinia deletion
mutant, vP723, which was isolated as a clear plaque in the
presence of X-gal.

**Example 5 - CONSTRUCTION OF PLASMID pMPCSK1Δ
               FOR DELETION OF OPEN READING FRAMES [C7L-K1L]**

    Referring now to FIG. 5, the following vaccinia clones
were utilized in the construction of pMPCSK1Δ. pSD420 is
SalI H cloned into pUC8. pSD435 is KpnI F cloned into
pUC18. pSD435 was cut with SphI and religated, forming
pSD451. In pSD451, DNA sequences to the left of the SphI
site (pos. 27,416) in HindIII M are removed (Perkus et al.,
1990). pSD409 is HindIII M cloned into pUC8.

    To provide a substrate for the deletion of the [C7L-
K1L] gene cluster from vaccinia, E. coli Beta-galactosidase
was first inserted into the vaccinia M2L deletion locus (Guo
et al., 1990) as follows. To eliminate the BglII site in
pSD409, the plasmid was cut with BglII in vaccinia sequences
(pos. 28,212) and with BamHI at the pUC/vaccinia junction,
then ligated to form plasmid pMP409B. pMP409B was cut at
the unique SphI site (pos. 27,416). M2L coding sequences
were removed by mutagenesis (Guo et al., 1990; Mandecki,
1986) using synthetic oligonucleotide

```
                                          BglII
MPSYN82 (SEQ ID NO:19) 5' TTTCTGTATATTTGCACCAATTTAGATCTT-
                          ACTCAAAATATGTAACAATA 3'
```

The resulting plasmid, pMP409D, contains a unique BglII site
inserted into the M2L deletion locus as indicated above. A
3.2 kb BamHI (partial)/BglII cassette containing the E. coli
Beta-galactosidase gene (Shapira et al., 1983) under the
control of the 11 kDa promoter (Bertholet et al., 1985) was
inserted into pMP409D cut with BglII. The resulting
plasmid, pMP409DBG (Guo et al., 1990), was used as donor
plasmid for recombination with rescuing vaccinia virus
vP723. Recombinant vaccinia virus, vP784, containing Beta-
galactosidase inserted into the M2L deletion locus, was
isolated as a blue plaque in the presence of X-gal.

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A plasmid deleted for vaccinia genes [C7L-K1L] was
assembled in pUC8 cut with SmaI, HindIII and blunt ended
with Klenow fragment of E. coli polymerase. The left
flanking arm consisting of vaccinia HindIII C sequences was
obtained by digestion of pSD420 with XbaI (pos. 18,628)
followed by blunt ending with Klenow fragment of E. coli
polymerase and digestion with BglII (pos. 19,706). The
right flanking arm consisting of vaccinia HindIII K
sequences was obtained by digestion of pSD451 with BglII
(pos. 29,062) and EcoRV (pos. 29,778). The resulting
plasmid, pMP581CK is deleted for vaccinia sequences between
the BglII site (pos. 19,706) in HindIII C and the BglII site
(pos. 29,062) in HindIII K. The site of the deletion of
vaccinia sequences in plasmid pMP581CK is indicated by a
triangle in FIG. 5.

To remove excess DNA at the vaccinia deletion junction,
plasmid pMP581CK, was cut at the NcoI sites within vaccinia
sequences (pos. 18,811; 19,655), treated with Bal-31
exonuclease and subjected to mutagenesis (Mandecki, 1986)
using synthetic oligonucleotide MPSYN233 (SEQ ID NO:20)
5'-TGTCATTTAACACTATACTCATATTAATAAAAATAATATTTATT-3'.
The resulting plasmid, pMPCSK1Δ, is deleted for vaccinia
sequences positions 18,805-29,108, encompassing 12 vaccinia
open reading frames [C7L - K1L]. Recombination between
pMPCSK1Δ and the Beta-galactosidase containing vaccinia
recombinant, vP784, resulted in vaccinia deletion mutant,
vP804, which was isolated as a clear plaque in the presence
of X-gal.

**Example 6   -   CONSTRUCTION OF PLASMID pSD548 FOR DELETION OF
                   LARGE SUBUNIT, RIBONUCLEOTIDE REDUCTASE (I4L)**

Referring now to FIG. 6, plasmid pSD405 contains
vaccinia HindIII I (pos. 63,875-70,367) cloned in pUC8.
pSD405 was digested with EcoRV within vaccinia sequences
(pos. 67,933) and with SmaI at the pUC/vaccinia junction,
and ligated, forming plasmid pSD518. pSD518 was used as the
source of all the vaccinia restriction fragments used in the
construction of pSD548.

The vaccinia I4L gene extends from position 67,371-
65,059. Direction of transcription for I4L is indicated by

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an arrow in FIG. 6. To obtain a vector plasmid fragment
deleted for a portion of the I4L coding sequences, pSD518
was digested with BamHI (pos. 65,381) and HpaI (pos. 67,001)
and blunt ended using Klenow fragment of E. coli polymerase.
This 4.8 kb vector fragment was ligated with a 3.2 kb SmaI
cassette containing the E. coli Beta-galactosidase gene
(Shapira et al., 1983) under the control of the vaccinia 11
kDa promoter (Bertholet et al., 1985; Perkus et al., 1990),
resulting in plasmid pSD524KBG. pSD524KBG was used as donor
plasmid for recombination with vaccinia virus vP804.
Recombinant vaccinia virus, vP855, containing Beta-
galactosidase in a partial deletion of the I4L gene, was
isolated as a blue plaque in the presence of X-gal.

      To delete Beta-galactosidase and the remainder of the
I4L ORF from vP855, deletion plasmid pSD548 was constructed.
The left and right vaccinia flanking arms were assembled
separately in pUC8 as detailed below and presented
schematically in FIG. 6.

      To construct a vector plasmid to accept the left
vaccinia flanking arm, pUC8 was cut with BamHI/EcoRI and
ligated with annealed synthetic oligonucleotides 518A1/518A2
(SEQ ID NO:21/SEQ ID NO:22)

```
           BamHI    RsaI
518A1  5' GATCCTGAGTACTTTGTAATATAATGATATATATTTTCACTTTATCTCAT
518A2  3'     GACTCATGAAACATTATATTACTATATATAAAAGTGAAATAGAGTA

                  BglII    EcoRI
           TTGAGAATAAAAAGATCTTAGG       3'     518A1
           AACTCTTATTTTTCTAGAATCCTTAA 5'      518A2
```

forming plasmid pSD531. pSD531 was cut with RsaI (partial)
and BamHI and a 2.7 kb vector fragment isolated. pSD518 was
cut with BglII (pos. 64,459)/ RsaI (pos. 64,994) and a 0.5
kb fragment isolated. The two fragments were ligated
together, forming pSD537, which contains the complete
vaccinia flanking arm left of the I4L coding sequences.

      To construct a vector plasmid to accept the right
vaccinia flanking arm, pUC8 was cut with BamHI/EcoRI and

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ligated with annealed synthetic oligonucleotides 518B1/518B2
(SEQ ID NO:23/SEQ ID NO:24)

```
          BamHI BglII SmaI
518B1  5' GATCCAGATCTCCCGGGAAAAAAATTATTTAACTTTTCATTAATAG-
518B2  3'      GTCTAGAGGGCCCTTTTTTTAATAAATTGAAAAGTAATTATC-

                  RsaI    EcoRI
     GGATTTGACGTATGTAGCGTACTAGG    3'    518B1
     CCTAAACTGCATACTACGCATGATCCTTAA 5'    518B2
```

forming plasmid pSD532. pSD532 was cut with RsaI
(partial)/EcoRI and a 2.7 kb vector fragment isolated.
pSD518 was cut with RsaI within vaccinia sequences (pos.
67,436) and EcoRI at the vaccinia/pUC junction, and a 0.6 kb
fragment isolated. The two fragments were ligated together,
forming pSD538, which contains the complete vaccinia
flanking arm to the right of I4L coding sequences.

The right vaccinia flanking arm was isolated as a 0.6
kb EcoRI/BglII fragment from pSD538 and ligated into pSD537
vector plasmid cut with EcoRI/BglII. In the resulting
plasmid, pSD539, the I4L ORF (pos. 65,047-67,386) is
replaced by a polylinker region, which is flanked by 0.6 kb
vaccinia DNA to the left and 0.6 kb vaccinia DNA to the
right, all in a pUC background. The site of deletion within
vaccinia sequences is indicated by a triangle in FIG. 6. To
avoid possible recombination of Beta-galactosidase sequences
in the pUC-derived portion of pSD539 with Beta-galactosidase
sequences in recombinant vaccinia virus vP855, the vaccinia
I4L deletion cassette was moved from pSD539 into pRC11, a
pUC derivative from which all Beta-galactosidase sequences
have been removed and replaced with a polylinker region
(Colinas et al., 1990). pSD539 was cut with EcoRI/PstI and
the 1.2 kb fragment isolated. This fragment was ligated
into pRC11 cut with EcoRI/PstI (2.35 kb), forming pSD548.
Recombination between pSD548 and the Beta-galactosidase
containing vaccinia recombinant, vP855, resulted in vaccinia
deletion mutant vP866, which was isolated as a clear plaque
in the presence of X-gal.

DNA from recombinant vaccinia virus vP866 was analyzed
by restriction digests followed by electrophoresis on an
agarose gel. The restriction patterns were as expected.
Polymerase chain reactions (PCR) (Engelke et al., 1988)

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using vP866 as template and primers flanking the six
deletion loci detailed above produced DNA fragments of the
expected sizes. Sequence analysis of the PCR generated
fragments around the areas of the deletion junctions
confirmed that the junctions were as expected. Recombinant
vaccinia virus vP866, containing the six engineered
deletions as described above, was designated vaccinia
vaccine strain "NYVAC."

**Example 7 - INSERTION OF A RABIES GLYCOPROTEIN
                G GENE INTO NYVAC**

The gene encoding rabies glycoprotein G under the
control of the vaccinia H6 promoter (Taylor et al., 1988a,b)
was inserted into TK deletion plasmid pSD513. pSD513 is
identical to plasmid pSD460 (FIG. 1) except for the presence
of a polylinker region.

Referring now to FIG. 7, the polylinker region was
inserted by cutting pSD460 with SmaI and ligating the
plasmid vector with annealed synthetic oligonucleotides
VQ1A/VQ1B (SEQ ID NO:25/SEQ ID NO:26)

```
         SmaI BglII XhoI  PstI  NarI  BamHI
VQ1A  5'  GGGAGATCTCTCGAGCTGCAGGGCGCCGGATCCTTTTTCT   3'
VQ1B  3'  CCCTCTAGAGAGCTCGACGTCCCGCGGCCTAGGAAAAAGA   5'
```

to form vector plasmid pSD513. pSD513 was cut with SmaI and
ligated with a SmaI ended 1.8 kb cassette containing the
gene encoding the rabies glycoprotein G gene under the
control of the vaccinia H6 promoter (Taylor et al.,
1988a,b). The resulting plasmid was designated pRW842.
pRW842 was used as donor plasmid for recombination with
NYVAC rescuing virus (vP866). Recombinant vaccinia virus
vP879 was identified by plaque hybridization using [32]P-
labelled DNA probe to rabies glycoprotein G coding
sequences.

The modified recombinant viruses of the present
invention provide advantages as recombinant vaccine vectors.
The attenuated virulence of the vector advantageously
reduces the opportunity for the possibility of a runaway
infection due to vaccination in the vaccinated individual
and also diminishes transmission from vaccinated to
unvaccinated individuals or contamination of the
environment.

The modified recombinant viruses are also advantageously used in a method for expressing a gene product in a cell cultured *in vitro* by introducing into the cell the modified recombinant virus having foreign DNA which codes for and expresses gene products in the cell.

Example 8 -   **CONSTRUCTION OF TROVAC-NDV EXPRESSING THE FUSION AND HEMAGGLUTININ-NEURAMINIDASE GLYCOPROTEINS OF NEWCASTLE DISEASE VIRUS**

A fowlpox virus (FPV) vector expressing both F and HN genes of the virulent NDV strain Texas was constructed. The recombinant produced was designated TROVAC-NDV. TROVAC-NDV expresses authentically processed NDV glycoproteins in avian cells infected with the recombinant virus and inoculation of day old chicks protects against subsequent virulent NDV challenge.

Cells and Viruses. The Texas strain of NDV is a velogenic strain. Preparation of cDNA clones of the F and HN genes has been previously described (Taylor et al., 1990; Edbauer et al., 1990). The strain of FPV designated FP-1 has been described previously (Taylor et al., 1988a). It is an attenuated vaccine strain useful in vaccination of day old chickens. The parental virus strain Duvette was obtained in France as a fowlpox scab from a chicken. The virus was attenuated by approximately 50 serial passages in chicken embryonated eggs followed by 25 passages on chicken embryo fibroblast cells. The virus was subjected to four successive plaque purifications. One plaque isolate was further amplified in primary CEF cells and a stock virus, designated as TROVAC, established. The stock virus used in the *in vitro* recombination test to produce TROVAC-NDV had been subjected to twelve passages in primary CEF cells from the plaque isolate.

Construction of a Cassette for NDV-F. A 1.8 kbp BamHI fragment containing all but 22 nucleotides from the 5' end of the F protein coding sequence was excised from pNDV81 (Taylor et al., 1990) and inserted at the BamHI site of pUC18 to form pCE13. The vaccinia virus H6 promoter previously described (Taylor et al., 1988a,b; Guo et al., 1989; Perkus et al., 1989) was inserted into pCE13 by digesting pCE13 with SalI, filling in the sticky ends with

Klenow fragment of E. coli DNA polymerase and digesting with
HindIII. A HindIII - EcoRV fragment containing the H6
promoter sequence was then inserted into pCE13 to form
pCE38. A perfect 5' end was generated by digesting pCE38
with KpnI and NruI and inserting the annealed and kinased
oligonucleotides CE75 (SEQ ID NO:27) and CE76 (SEQ ID NO:28)
to generate pCE47.

CE75:
CGATATCCGTTAAGTTTGTATCGTAATGGGCTCCAGATCTTCTACCAGGATCCCGGTAC

CE76:
CGGGATCCTGGTAGAAGATCTGGAGCCCATTACGATACAAACTTAACGGATATCG.

In order to remove non-coding sequence from the 3' end of
the NDV-F from a SmaI to PstI fragment from pCE13 was inserted
into the SmaI and PstI sites of pUC18 to form pCE23. The
non-coding sequences were removed by sequential digestion of
pCE23 with SacI, BamHI, Exonuclease III, SI nuclease and
EcoRI. The annealed and kinased oligonucleotides CE42 (SEQ
ID NO:29) and CE43 (SEQ ID NO:30) were then inserted to form
pCE29.

CE42: AATTCGAGCTCCCCGGG

CE43: CCCGGGGAGCTCG

The 3' end of the NDV-F sequence was then inserted into
plasmid pCE20 already containing the 5' end of NDV-F by
cloning a PstI - SacI fragment from pCE29 into the PstI and
SacI sites of pCE20 to form pCE32. Generation of pCE20 has
previously been described in Taylor et al., 1990.

    In order to align the H6 promoter and NDV-F 5'
sequences contained in pCE47 with the 3' NDV-F sequences
contained in pCE32, a HindIII - PstI fragment of pCE47 was
inserted into the HindIII and PstI sites of pCE32 to form
pCE49. The H6 promoted NDV-F sequences were then
transferred to the de-ORFed F8 locus (described below) by
cloning a HindIII - NruI fragment from pCE49 into the
HindIII and SmaI sites of pJCA002 (described below) to form
pCE54. Transcription stop signals were inserted into pCE54
by digesting pCE54 with SacI, partially digesting with BamHI
and inserting the annealed and kinased oligonucleotides
CE166 (SEQ ID NO:31) and CE167 (SEQ ID NO:32) to generate
pCE58.

CE166:　CTTTTTATAAAAAGTTAACTACGTAG

CE167:　GATCCTACGTAGTTAACTTTTTATAAAAAGAGCT

A perfect 3' end for NDV-F was obtained by using the
polymerase chain reaction (PCR) with pCE54 as template and
oligonucleotides CE182 (SEQ ID NO:33) and CE183 (SEQ ID
NO:34) as primers.

CE182:　CTTAACTCAGCTGACTATCC

CE183:　TACGTAGTTAACTTTTTATAAAAATCATATTTTTGTAGTGGCTC

The PCR fragment was digested with PvuII and HpaI and cloned
into pCE58 that had been digested with HpaI and partially
digested with PvuII. The resulting plasmid was designated
pCE64. Translation stop signals were inserted by cloning a
HindIII - HpaI fragment which contains the complete H6
promoter and F coding sequence from pCE64 into the HindIII
and HpaI sites of pRW846 to generate pCE71, the final
cassette for NDV-F. Plasmid pRW846 is essentially
equivalent to plasmid pJCA002 (described below) but
containing the H6 promoter and transcription and translation
stop signals. Digestion of pRW846 with HindIII and HpaI
eliminates the H6 promoter but leaves the stop signals
intact.

　　Construction of Cassette for NDV-HN. Construction of
plasmid pRW802 was previously described in Edbauer et al.,
1990. This plasmid contains the NDV-HN sequences linked to
the 3' end of the vaccinia virus H6 promoter in a pUC9
vector. A HindIII - EcoRV fragment encompassing the 5' end
of the vaccinia virus H6 promoter was inserted into the
HindIII and EcoRV sites of pRW802 to form pRW830. A perfect
3' end for NDV-HN was obtained by inserting the annealed and
kinased oligonucleotides CE162
(SEQ ID NO:35) and CE163 (SEQ ID NO:36) into the EcoRI site
of pRW830 to form pCE59, the final cassette for NDV-HN.

CE162:
AATTCAGGATCGTTCCTTTACTAGTTGAGATTCTCAAGGATGATGGGATTTAATTTTTAT
AAGCTTG

CE163:
AATTCAAGCTTATAAAAATTAAATCCCATCATCCTTGAGAATCTCAACTAGTAAAGGAAC
GATCCTG

     Construction of FPV Insertion Vector. Plasmid pRW731-
15 contains a 10kb PvuII - PvuII fragment cloned from
genomic DNA. The nucleotide sequence was determined on both
strands for a 3660 bp PvuII - EcoRV fragment. The limits of
an open reading frame designated here as F8 were determined.
Plasmid pRW761 is a sub-clone of pRW731-15 containing a 2430
bp EcoRV - EcoRV fragment. The F8 ORF was entirely
contained between an XbaI site and an SspI site in pRW761.
In order to create an insertion plasmid which on
recombination with TROVAC genomic DNA would eliminate the F8
ORF, the following steps were followed. Plasmid pRW761 was
completely digested with XbaI and partially digested with
SspI. A 3700 bp XbaI - SspI band was isolated from the gel
and ligated with the annealed double-stranded
oligonucleotides JCA017 (SEQ ID NO:37) and JCA018 (SEQ ID
NO:38).
JCA017:5'
CTAGACACTTTATGTTTTTTAATATCCGGTCTTAAAAGCTTCCCGGGGATCCTTATACGG
GGAATAAT
JCA018:5'
ATTATTCCCCGTATAAGGATCCCCCGGGAAGCTTTTAAGACCGGATATTAAAAAACATAA
AGTGT
The plasmid resulting from this ligation was designated
pJCA002.
     Construction of Double Insertion Vector for NDV F and
HN. The H6 promoted NDV-HN sequence was inserted into the
H6 promoted NDV-F cassette by cloning a HindIII fragment
from pCE59 that had been filled in with Klenow fragment of
E. coli DNA polymerase into the HpaI site of pCE71 to form
pCE80. Plasmid pCE80 was completely digested with NdeI and
partially digested with BglII to generate an NdeI - BglII
4760 bp fragment containing the NDV F and HN genes both
driven by the H6 promoter and linked to F8 flanking arms.
Plasmid pJCA021 was obtained by inserting a 4900 bp PvuII -
HindII fragment from pRW731-15 into the SmaI and HindII
sites of pBSSK+. Plasmid pJCA021 was then digested with
NdeI and BglII and ligated to the 4760 bp NdeI - BglII
fragment of pCE80 to form pJCA024. Plasmid pJCA024
therefore contains the NDV-F and HN genes inserted in

opposite orientation with 3' ends adjacent between FPV
flanking arms. Both genes are linked to the vaccinia virus
H6 promoter. The right flanking arm adjacent to the NDV-F
sequence consists of 2350 bp of FPV sequence. The left
flanking arm adjacent to the NDV-HN sequence consists of
1700 bp of FPV sequence.

Development of TROVAC-NDV. Plasmid pJCA024 was
transfected into TROVAC infected primary CEF cells by using
the calcium phosphate precipitation method previously
described (Panicali et al., 1982; Piccini et al., 1987).
Positive plaques were selected on the basis of hybridization
to specific NDV-F and HN radiolabelled probes and subjected
to five sequential rounds of plaque purification until a
pure population was achieved. One representative plaque was
then amplified and the resulting TROVAC recombinant was
designated TROVAC-NDV (vFP96).

Immunofluorescence. Indirect immunofluorescence was
performed as described (Taylor et al., 1990) using a
polyclonal anti-NDV serum and, as mono-specific reagents,
sera produced in rabbits against vaccinia virus recombinants
expressing NDV-F or NDV-HN.

Immunoprecipitation. Immunoprecipitation reactions
were performed as described (Taylor et al., 1990) using a
polyclonal anti-NDV serum obtained from SPAFAS Inc., Storrs,
CN.

The stock virus was screened by in situ plaque
hybridization to confirm that the F8 ORF was deleted. The
correct insertion of the NDV genes into the TROVAC genome
and the deletion of the F8 ORF was also confirmed by
Southern blot hybridization.

In NDV-infected cells, the F glycoprotein is anchored
in the membrane via a hydrophobic transmembrane region near
the carboxyl terminus and requires post-translational
cleavage of a precursor, F_0, into two disulfide linked
polypeptides F_1 and F_2. Cleavage of F_0 is important in
determining the pathogenicity of a given NDV strain (Homma
and Ohuchi, 1973; Nagai et al., 1976; Nagai et al., 1980),
and the sequence of amino acids at the cleavage site is
therefore critical in determining viral virulence. It has

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been determined that amino acids at the cleavage site in the
NDV-F sequence inserted into FPV to form recombinant vFP29
had the sequence Arg – Arg – Gln – Arg – Arg (SEQ ID NO:39)
(Taylor et al., 1990) which conforms to the sequence found
to be a requirement for virulent NDV strains (Chambers et
al., 1986; Espion et al., 1987; Le et al., 1988; McGinnes
and Morrison, 1986; Toyoda et al., 1987). The HN
glycoprotein synthesized in cells infected with virulent
strains of NDV is an uncleaved glycoprotein of 74 kDa.
Extremely avirulent strains such as Ulster and Queensland
encode an HN precursor (HNo) which requires cleavage for
activation (Garten et al., 1980).

    The expression of F and HN genes in TROVAC-NDV was
analyzed to confirm that the gene products were
authentically processed and presented. Indirect-
immunofluorescence using a polyclonal anti-NDV chicken serum
confirmed that immunoreactive proteins were presented on the
infected cell surface. To determine that both proteins were
presented on the plasma membrane, mono-specific rabbit sera
were produced against vaccinia recombinants expressing
either the F or HN glycoproteins. Indirect
immunofluorescence using these sera confirmed the surface
presentation of both proteins.

    Immunoprecipitation experiments were performed by using
(^{35}S) methionine labeled lysates of CEF cells infected with
parental and recombinant viruses. The expected values of
apparent molecular weights of the glycolysated forms of F_1
and F_2 are 54.7 and 10.3 kDa respectively (Chambers et al.,
1986). In the immunoprecipitation experiments using a
polyclonal anti-NDV serum, fusion specific products of the
appropriate size were detected from the NDV-F single
recombinant vFP29 (Taylor et al., 1990) and the TROVAC-NDV
double recombinant vFP96. The HN glycoprotein of
appropriate size was also detected from the NDV-HN single
recombinant VFP-47 (Edbauer et al., 1990) and TROVAC-NDV.
No NDV specific products were detected from uninfected and
parental TROVAC infected CEF cells.

    In CEF cells, the F and HN glycoproteins are
appropriately presented on the infected cell surface where

they are recognized by NDV immune serum.
Immunoprecipitation analysis indicated that the F_0 protein
is authentically cleaved to the F_1 and F_2 components
required in virulent strains. Similarly, the HN
glycoprotein was authentically processed in CEF cells
infected with recombinant TROVAC-NDV.

     Previous reports (Taylor et al., 1990; Edbauer et al.,
1990; Boursnell et al., 1990a,b,c; Ogawa et al., 1990) would
indicate that expression of either HN or F alone is
sufficient to elicit protective immunity against NDV
challenge. Work on other paramyxoviruses has indicated,
however, that antibody to both proteins may be required for
full protective immunity. It has been demonstrated that SV5
virus could spread in tissue culture in the presence of
antibody to the HN glycoprotein but not to the F
glycoprotein (Merz et al., 1980). In addition, it has been
suggested that vaccine failures with killed measles virus
vaccines were due to inactivation of the fusion component
(Norrby et al., 1975). Since both NDV glycoproteins have
been shown to be responsible for eliciting virus
neutralizing antibody (Avery et al., 1979) and both
glycoproteins, when expressed individually in a fowlpox
vector are able to induce a protective immune response, it
can be appreciated that the most efficacious NDV vaccine
should express both glycoproteins.

Example 9 -   **CONSTRUCTION OF NYVAC-MV RECOMBINANT**
                   EXPRESSINGMEASLES FUSION AND HEMAGGLUTININ
                   GLYCOPROTEINS

     cDNA copies of the sequences encoding the HA and F
proteins of measles virus MV (Edmonston strain) were
inserted into NYVAC to create a double recombinant
designated NYVAC-MV. The recombinant authentically
expressed both measles glycoproteins on the surface of
infected cells. Immunoprecipitation analysis demonstrated
correct processing of both F and HA glycoproteins. The
recombinant was also shown to induce syncytia formation.

     Cells and Viruses. The rescuing virus used in the
production of NYVAC-MV was the modified Copenhagen strain of
vaccinia virus designated NYVAC. All viruses were grown and
titered on Vero cell monolayers.

Plasmid Construction. Plasmid pSPM2LHA (Taylor et al.,
1991c) contains the entire measles HA gene linked in a
precise ATG to ATG configuration with the vaccinia virus H6
promoter which has been previously described (Taylor et al.,
1988a,b; Guo et al., 1989; Perkus et al., 1989). A 1.8kpb
EcoRV/SmaI fragment containing the 3' most 24 bp of the H6
promoter fused in a precise ATG:ATG configuration with the
HA gene lacking the 3' most 26 bp was isolated from
pSPM2LHA. This fragment was used to replace the 1.8 kbp
EcoRV/SmaI fragment of pSPMHHA11 (Taylor et al., 1991c) to
generate pRW803. Plasmid pRW803 contains the en- tire H6
promoter linked precisely to the entire measles HA gene.

In the confirmation of previous constructs with the
measles HA gene it was noted that the sequence for codon
18(CCC) was deleted as compared to the published sequence
(Alkhatib et al., 1986). The CCC sequence was replaced by
oligonucleotide mutagenesis via the Kunkel method (Kunkel,
1985) using oligonucleotide RW117 (SEQ ID NO:40)
(5'GACTATCCTACTTCCCTTGGGATGGGGGTTATCTTTGTA-3').

PRO 18

Single stranded template was derived from plasmid pRW819
which contains the H6/HA cassette from pRW803 in pIBI25
(International Biotechnologies, Inc., New Haven, CT). The
mutagenized plasmid containing the inserted (CCC) to encode
for a proline residue at codon 18 was designated pRW820.
The sequence between the HindIII and XbaI sites of pRW820
was confirmed by nucleotide sequence analysis. The HindIII
site is situated at the 5' border of the H6 promoter while
the XbaI site is located 230 bp downstream from the
initiation codon of the HA gene. A 1.6 kbp XbaI/EcoRI
fragment from pRW803, containing the HA coding sequences
downstream from the XbaI (above) and including the
termination codon, was used to replace the equivalent
fragment of pRW820 resulting in the generation of pRW837.
The mutagenized expression cassette contained within pRW837
was derived by digestion with HindIII and EcoRI, blunt-ended
using the Klenow fragment of E. coli DNA polymerase in the
presence of 2mM dNTPs, and inserted into the SmaI site of
pSD513 to yield pRW843. Plasmid pSD513 was derived from

plasmid pSD460 by the addition of polylinker sequences.
Plasmid pSD460 was derived to enable deletion of the
thymidine kinase gene from vaccinia virus.

To insert the measles virus F gene into the HA
insertion plasmid, manipulations were performed on pSPHMF7.
Plasmid pSPHMF7 (Taylor et al., 1991c) contains the measles
F gene juxtaposed 3' to the previously described vaccinia
virus H6 promoter. In order to attain a perfect ATG for ATG
configuration and remove intervening sequences between the
3' end of the promoter and the ATG of the measles F gene
oligonucleotide directed mutagenesis was performed using
oligonucleotide SPMAD (SEQ ID NO:41).
SPMAD: 5'- TATCCGTTAAGTTTGTATCGTAATGGGTCTCAAGGTGAACGTCT-3'
The resultant plasmid was designated pSPMF75M20.

The plasmid pSPMF75M20 which contains the measles F
gene now linked in a precise ATG for ATG configuration with
the H6 promoter was digested with NruI and EagI. The
resulting 1.7 kbp blunt ended fragment containing the 3'
most 27 bp of the H6 promoter and the entire fusion gene was
isolated and inserted into an intermediate plasmid pRW823
which had been digested with NruI and XbaI and blunt ended.
The resultant plasmid pRW841 contains the H6 promoter linked
to the measles F gene in the pIBI25 plasmid vector
(International Biotechnologies, Inc., New Haven, CT). The
H6/measles F cassette was excised from pRW841 by digestion
with SmaI and the resulting 1.8 kb fragment was inserted
into pRW843 (containing the measles HA gene). Plasmid
pRW843 was first digested with NotI and blunt-ended with
Klenow fragment of E. coli DNA polymerase in the presence of
2mM dNTPs. The resulting plasmid, pRW857, therefore
contains the measles virus F and HA genes linked in a tail
to tail configuration. Both genes are linked to the
vaccinia virus H6 promoter.

Development of NYVAC-MV. Plasmid pRW857 was
transfected into NYVAC infected Vero cells by using the
calcium phosphate precipitation method previously described
(Panicali et al., 1982; Piccini et al., 1987). Positive
plaques were selected on the basis of in situ plaque
hybridization to specific MV F and HA radiolabelled probes

and subjected to 6 sequential rounds of plaque purification
until a pure population was achieved. One representative
plaque was then amplified and the resulting recombinant was
designated NYVAC-MV (vP913).

    Immunofluorescence. Indirect immunofluorescence was
performed as previously described (Taylor et al., 1990).
Mono-specific reagents used were sera generated by
inoculation of rabbits with canarypox recombinants
expressing either the measles F or HA genes.

    Immunoprecipitation. Immunoprecipitation reactions
were performed as previously described (Taylor et al., 1990)
using a guinea-pig anti measles serum (Whittaker M.A.
Bioproducts, Walkersville, MD).

    Cell Fusion Experiments. Vero cell monolayers in 60mm
dishes were inoculated at a multiplicity of 1 pfu per cell
with parental or recombinant viruses. After 1 h absorption
at 37°C the inoculum was removed, the overlay medium
replaced and the dishes inoculated overnight at 37°C. At 20
h post-infection, dishes were examined.

    In order to determine that the expression products of
both measles virus F and HA genes were presented on the
infected cell surface, indirect immunofluorescence analysis
was performed using mono-specific sera generated in rabbits
against canarypox recombinants expressing either the measles
F or HA genes. The results indicated that both F and HA
gene products were expressed on the infected cell surface,
as demonstrated by strong surface fluorescence with both
mono-specific sera. No background staining was evident with
either sera on cells inoculated with the parental NYVAC
strain, nor was there cross-reactive staining when mono-
specific sera were tested against vaccinia single
recombinants expressing either the HA or F gene.

    In order to demonstrate that the proteins expressed by
NYVAC-MV were immunoreactive with measles virus specific
sera and were authentically processed in the infected cell,
immunoprecipitation analysis was performed. Vero cell
monolayers were inoculated at a multiplicity of 10 pfu/cell
of parental or recombinant viruses in the presence of ^{35}s-
methionine. Immunoprecipitation analysis revealed a HA

glycoprotein of approximately 76 kDa and the cleaved fusion
products F_1 and F_2 with molecular weights of 44 kDa and 23
kDa, respectively. No measles specific products were
detected in uninfected Vero cells or Vero cells infected
with the parental NYVAC virus.

     A characteristic of MV cytopathology is the formation
of syncytia which arise by fusion of infected cells with
surrounding infected or uninfected cells followed by
migration of the nuclei toward the center of the syncytium
(Norrby et al., 1982). This has been shown to be an
important method of viral spread, which for Paramyxoviruses,
can occur in the presence of HA-specific virus neutralizing
antibody (Merz et al., 1980). In order to determine that
the MV proteins expressed in vaccinia virus were
functionally active, Vero cell monolayers were inoculated
with NYVAC and NYVAC-MV and observed for cytopathic effects.
Strong cell fusing activity was evident in NYVAC-MV infected
Vero cells at approximately 18 hours post infection. No
cell fusing activity was evident in cells infected with
parental NYVAC.

**Example 10 - CONSTRUCTION OF NYVAC RECOMBINANTS EXPRESSING
             GLYCOPROTEINS OF PSEUDORABIES VIRUS**

     It has been demonstrated that vaccinia virus
recombinants expressing the PRV gpII, gpIII, and gp50
glycoproteins either individually or in combination provide
efficacious vaccine candidates, in that, they protect swine
from a virulent challenge with live PRV. Considering the
inability of the NYVAC vector to productively replicate in
porcine cell cultures and the inherent safety of the vector
due to the deletion of known potential virulence genes,
NYVAC-based recombinants containing the PRV gpII, gpIII, and
gp50 either alone or in various combinations have been
generated. These recombinants were generated to provide
efficacious vaccine candidates against PRV that were safe
for swine and eliminated or severely limited transmission to
the environment.

     Viruses and Cells. Manipulations of NYVAC and
molecular cloning were performed by standard techniques
(Piccini et al., 1987; Maniatis et al., 1982). Cultivation

of NYVAC and NYVAC-based recombinants was as previously
described (Piccini et al., 1987).

Cloning of the PRV gpII, gpIII, and gp50 Genes. The
growth of PRV, extraction of PRV genomic DNA, and the
identification of the PRV gpII, gpIII, and gp50 genes have
been described.

Cloning and Expression of the Pseudorabies Virus (PRV)
Genes into NYVAC (vP866). The NYVAC deletion mutant lacking
a region encompassing the human and porcine host range genes
(C7L and K1L), vP866, was the basic vector used to insert
the PRV genes. This vector also lacks the vaccinia virus tk
gene, hemagglutinin gene, hemorrhagic gene, ribonucleotide
reductase (large subunit) gene, and A-type inclusion gene.
Importantly, vP866 does not replicate efficiently, if at
all, on human or pig kidney (LLC-PK1) cells. PRV genes
gpII, gpIII, and gp50, which are homologous to the herpes
simplex virus gB (Robbins et al., 1987), gC (Robbins et al.,
1986b), and gD (Wathen and Wathen, 1984), respectively, were
inserted into vP866 as outlined below.

Insertion of the PRV gpII Gene into the Hemagglutinin
Locus of vP866. The DNA sequence encoding the PRV gpII gene
resides in the BamHI fragment 1 of the PRV genome
(Mettenleiter et al., 1986).

The plasmid designated pPR9.25, containing the PRV
BamHI fragment 1 inserted into the BamHI site of pBR322 was
digested with NcoI. The resultant restriction fragments
were fractionated on a 0.8% agarose gel and a 6.2 kb NcoI
DNA fragment was purified using Geneclean (Bio101, Inc.,
LaJolla, CA) and subsequently inserted into the NcoI site of
pBR328 (Boehringer Mannheim Biochemicals, Indianapolis, IN)
treated with CiAP. The resulting plasmid, pPR2.15, was
digested with SphI and fractionated on an agarose gel. The
2.7 kb and 1.8 kb fragments were purified and inserted into
the SphI site of pUC18 to create pPR1 and pPR2,
respectively.

The 1060 bp PRV SphI/NheI fragment from pPR1 was
isolated from an agarose gel and inserted into the
BamHI/SphI sites of pIBI25 with annealed oligonucleotides
MRSYN1 (SEQ ID NO:42) (5'-GATCCATTCCATGGTTG-3') and MRSYN2

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(SEQ ID NO:43) (5'-TAGCAACCATGGAATG-3') to generate pPR6.
pPR6 was digested with HindIII and ApaI. The ApaI site is
located 32 bp downstream from the ATG initiation codon of
PRV gpII. This 3920 bp fragment was ligated to annealed
oligonucleotides MRSYN3 (SEQ ID NO:44) (5'-
AGCTTGATATCCGTTAAGTTTGTATCGTAATGCCCGCTGGTGGCGGTCTTTGGCGCGGGC
C-3') and MRSYN4 (SEQ ID NO:45) (5'-
CGCGCCAAAGACCGCCAACCAGCGGGATTACGATACAAACTTAACGGATATCA-3') to
generate pPR9. These annealed oligonucleotides provide the
DNA sequences specifying the vaccinia virus H6 promoter from
the EcoRV site through the ATG, followed by the PRV gpII
coding sequences. The plasmid pPR9 was digested with BamHI
and NheI and treated with Calf Intestinal Alkaline
Phosphatase (CiAP), and ligated to annealed oligonucleotides
MRSYN7 (SEQ ID NO:46) (5'-CCCAGATCTCCTTG-3') and MRSYN8 (SEQ
ID NO:47) (5'-GTACGGGTCTAGAGGAACCTAG-3') and a 1640 bp
SphI/NheI fragment obtained from pPR1 generating plasmid
pPR12.

The 1030 bp HindII/SphI fragment from pPR2 was isolated
from an agarose gel and inserted into a HincII/SphI pUC18
vector. The resulting plasmid, pPR10, was digested with
HindIII and NaeI and treated with CiAP. The NaeI site is
located 44 bp upstream of the termination codon (TAG).
Annealed oligonucleotides MRSYN9 (SEQ ID NO:48) (5'-GGCACT
ACCAGCGCCTCGAGAGCGAGGACCCCGACGCCCTGTAGAATTTTTATCGGCCGA-3')
and MRSYN10 (SEQ ID NO:49) (5'-AGCTTCGGCCGATAAAAATTCTA
CAGGGCGTCGGGGTCCTCGCTCTCGAGGCGTAGTGCC-3') were ligated to
the 3720 bp NaeI/HindIII fragment of pPR10 to yield plasmid
pPR11. A 770 bp SphI/HincII fragment from pPR2 was purified
from an agarose gel and inserted using the BamHI/SphI
phosphorylated linker MRSYN7 (SEQ ID NO:46) and MRSYN8 (SEQ
ID NO:47) into the BamHI/HincII sites of CiAP treated pPR11
to generate pPR13. Plasmid pPR12 digested with EcoRI and
SphI was ligated using MRSYN19 (SEQ ID NO:50) (5'-AGCTTCTGC
AGCCATGGCGATCGG-3') and MRSYN20 (SEQ ID NO:51) (5'-AATTCCG
ATCGCCATGGCTGCAGA-3') to a 990 bp HindIII/SphI fragment from
pPR13 to yield plasmid pPR15. Plasmid, pPR15, was digested
with HindIII/EcoRV to yield a 2780 bp fragment. This
fragment was inserted into pTP15 (Guo et al., 1989) which

was digested with XmaIII and EcoRV to generate pPR18. In
pPR18, the PRVgpII is linked with the vaccinia virus H6
promoter in a HA deletion plasmid. pPR18 was used in *in
vitro* recombination experiments with vP866 as the rescue
virus to generate vP881.

     Insertion of the PRV gpIII gene into the TK Locus of
NYVAC. The sequences encoding the PRV gpIII gene map to the
BamHI 2 and 9 fragments of the PRV genome (Robbins et al.,
1986b). Plasmids pPR9.9 and pPR7.35 contain the PRV BamHI
fragments 2 and 9, respectively, inserted into the BamHI
site of pBR322. An SphI/BamHI fragment containing the 5'
end of the PRV gpIII gene was isolated from pPR9.9. An
NcoI/BamHI fragment containing the remainder of the gpIII
gene was isolated from pPR7.35. The entire PRV gpIII gene
was assembled by the ligation of these two fragments into
pIBI25 to yield pPR17.

     The PRV gpIII gene was manipulated to be expressed
under the control of the early vaccinia virus hemorrhagic
promoter, located in the HindIII B region (Goebel et al.,
1990a,b). Using site-directed mutagenesis, an NsiI site was
introduced by changing the sequence CGC (bases 192-194) in
PRV gpIII to ATG and an XbaI site was introduced by changing
the sequence GTCACGT to TTCTAGA (bases 1632-1638). To
perform the mutagenesis reactions, single-stranded DNA was
generated from plasmid pPR17 using the helper phage R408
(Stratagene, LaJolla, CA). The site-directed mutagenesis
was done using MRSYN5 (SEQ ID NO:52) (5'-GCGAGCGAGGCCATGC
ATCGTGCGAATGGCCCC-3') and MRSYN6 (SEQ ID NO:53) (5'-GGGGG
GACGCGCGGGTCTAGAAGGCCCCGCCTGGCGG-3') and selection on *E.
coli* dut⁻ ung⁻ strain. CJ236 (International
Biotechnologies, Inc., New Haven, CT). Mutagenesis was
performed according to the protocols of Kunkel (1985).
These mutations resulted in the generation of plasmid pPR28.

     Plasmid pPR28 was digested with NsiI and XbaI and
treated with Mung bean nuclease. A 1440 bp fragment was
purified and inserted into a BglII/HpaI digested pSD478VC
after treatment with Mung bean nuclease and calf-intestine
alkaline phosphatase. The resultant plasmid was designated
as pPR24.

The plasmid pPR24 was digested with SnaBl and DraI to liberate a 1500 bp blunt-ended fragment containing the u promoter and PRV gpIII gene. This fragment was ligated into SmaI digested pSD513VC to yield pPRVIIIVCTK. *In vitro* recombination experiments were performed with pPRVIIIVCTK and vP866 as the rescue virus to generate vP883. In vP883, the vaccinia tk coding sequences are replaced by the PRV gpIII gene inserted in a right to left orientation, with respect to the genome, under the control of the 120 bp vaccinia u promoter element.

    Insertion of the PRV gp50 Gene into the ATI Locus of NYVAC. DNA encoding the gene for the PRV glycoprotein gp50 is located on the BamHI fragment 7 of the PRV genome (Petrovskis et al., 1986a,b). Plasmid pPR7.1 contains the PRV BamHI fragment 7 inserted into the BamHI site of pBR322. A StuI/NdeI subfragment of pPR7.1 containing the entire gp50 gene was subcloned into pIBI25 to yield plasmid #856.

    The coding sequences for PRV gp50 were placed under the control of the early/intermediate vaccinia promoter, I3L (Schmitt and Stunnenberg, 1988; Vos and Stunnenberg, 1988). This promoter element has been used previously to express foreign genes in vaccinia recombinants (Perkus et al., 1985; Bucher et al., 1989). DNA corresponding to promoter sequences upstream from the I3L open reading frame (Schmitt and Stunnenberg, 1988) was derived by PCR (Saiki et al., 1988) using synthetic oligonucleotides P50PPBAM (SEQ ID NO:54) (5'-ATCATCGGATCCGGTGGTTTGCCATTCCG-3') and P50PPATG (SEQ ID NO:55) (5'-GATTAAACCTAAATAATTG-3') and pMPIVC, a subclone of the Copenhagen HindIII I region, as template. The resulting 126 bp fragment was digested with BamHI to generate a BamHI cohesive end at the 5' end of the promoter sequence. The 3' end remained blunt-ended.

    The PRV gp50 coding region was excised from plasmid #856. Plasmid #856 was initially digested with NsiI, which cuts 7 bp upstream from the ATG and results in a 3' overhang. The 3' overhang was blunt-ended with T4 DNA polymerase in the presence of 2mM dNTPs. The resulting DNA was partially digested with BglII, and a 1.3kb blunt/BglII fragment containing the PRV gp50 gene was isolated.

The 126 bp I3L promoter fragment (BamHI/blunt) and the
1.3kb gp50 gene containing fragment (blunt/BglII) were
ligated into pBS-SK (Stratagene, La Jolla, CA) digested with
BamHI. The resultant plasmid was designated as pBSPRV50I3.
The expression cassette containing the I3L promoter linked
to the PRV gp50 gene was excised by a BamHl/partial SmaI
digestion. A 1.4 kb fragment containing the I3L
promoter/PRV gp50 gene was isolated and blunt-ended using
the Klenow fragment of the E. coli DNA polymerase in the
presence of 2mM dNTPs.

The 1.4 kb blunt-ended fragment containing the I3L
promoter/PRV gp50 gene was inserted into the ATI insertion
plasmid pSD541. Flanking arms for the ATI region were
generated by PCR using subclones of the Copenhagen HindIII A
region as template. Oligonucleotides MPSYN267 (SEQ ID
NO:56) (5'-GGGCTGAAGCTTGCGGCCGCTCATTAGACAAGCGAATGAGGGAC-3')
and MPSYN268 (SEQ ID NO:57) (5'-
AGATCTCCCGGGCTCGAGTAATTAATTAATTTTTATTACACCAGAAAGACGGCTTGAGAT
C-3') were used to derive the 420 bp vaccinia arm to the
right of the ATI deletion. Synthetic oligonucleotides
MPSYN269 (SEQ ID NO:58) (5'-
TAATTACTGAGCCCGGGAGATATAATTTAATTTAATTTATATAACTCATTTTTTCCCC-
3') and MPSYN270 (SEQ ID NO:59) (5'-
TATCTCGAATTCCCGCGGCTTTAAATGGACGGAACTCTTTTCCC-3') were used
to derive the 420 bp vaccinia arm to the left of the
deletion. The left and right arms were fused together by
PCR and are separated by a polylinker region specifying
restriction sites for BglII, SmaI, and XhoI. The PCR-
generated fragment was digested with HindIII and EcoRI to
yield sticky ends, and ligated into pUC8 digested with
HindIII and EcoRI to generate pSD541.

The pSD541 plasmid containing the I3L/PRV gp50 gene was
designated as pATIgp50. This plasmid was used in *in vitro*
recombination experiments to generate vP900. vP900 contains
the PRV gp50 gene in place of the ATI gene.

Generation of Double and Triple PRV Recombinants in
NYVAC. *In vitro* recombination experiments were performed to
generate NYVAC-based recombinants containing multiple PRV
genes. *In vitro* recombination experiments using the donor

plasmid, pATIP50, were performed with vP881, vP883, and
vP915 to generate vP912, vP916, and vP925, respectively
(Table 1). Experiments were done with plasmid pPR18 and
vP883 as rescue virus to yield vP915 (Table 1).

    Immunoprecipitation from NYVAC/PRV Recombinant Infected
Cells. Vero cells were infected at an m.o.i. of 10 pfu per
cell with the individual recombinant viruses, with the NYVAC
parent virus, or were mock infected. After a 1 hr
absorption period, the inoculum was removed and infected
cells were overlaid with methionine-free media containing
^{35}S-methionine (20uCi/ml). All samples were harvested at 8
hr post infection. For samples analyzed with the sheep
.anti-gpII sera, cells were harvested by centrifugation and
were dissociated with RIPA buffer (1% NP-40, 1% Na-
deoxycholate, 0.1% SDS, 0.01M methionine, 5mM EDTA, 5mM 2-
mercapto-ethanol, 1m/ml BSA, and 100 u/ml aprotinin).
Samples analyzed with sheep anti-gpIII and a monoclonal
specific for gp50 were lysed in 1X Buffer A (1% NP40, 10mM
Tris (pH7.4), 150mM NaCl, 1mM EDTA, 0.0% Na Azide, 0.2mg/ml
PMSF). All sera was preadsorbed with vP866 infected Vero
cells and all lysates were preadsorbed with normal sera
(sheep or mouse) and protein A-sepharose linked to the
secondary antibody.

    Lysates from the infected cells were analyzed for PRV
gpII expression using a sheep anti-gpII serum. This primary
antiserum was incubated with protein A-sepharose conjugated
with rabbit anti-sheep IgG (Boehringer-Mannheim). After an
overnight incubation at 4°C, samples were washed 4 times
with 1 x RIPA buffer, 2 times with LiCl-urea (0.2M LiCl, 2M
urea, 10mM Tris, ph 8.0). Precipitates were harvested by
micro centrifugation. Precipitated protein was dissociated
from the immune complexes by the addition of 2X Laemmli's
·buffer (125 mM Tris (pH 6.8), 4% (SDS), 20% glycerol, 10% 2
mercapto-ethanol) and boiling for 5 min. Proteins were
fractionated on a 10% Dreyfuss gel system (Dreyfuss et al.,
1984), fixed and treated with 1M Na-Salicylate for
fluorography.

    Lysates were analyzed for PRV gpIII expression using a
sheep-anti gpIII sera. This primary antisera was incubated

with Protein A-Sepharose conjugated with rabbit anti-sheep
IgG (Boehringer-Mannhein). After an overnight incubation at
4°C, samples were washed 4 times with 1X Buffer A and 2
times with the LiCl-urea buffer. Precipitates were treated
and analyzed by flurography as described above.

    Lysates were analyzed for PRV gp50 expression using
monoclonal antibody, 22M4 (provided by Rhone Merieux, Lyon,
France). This primary antibody was incubated with Protein
A-Sepharose conjugated with goat anti-mouse IgG and IgM
(Boehringer-Mannheim). The precipitates were recovered and
analyzed as described above for the PRV gpIII
immunoprecipitations.

    Expression of the PRV Glycoproteins in Cells Infected
with the NYVAC/PRV Recombinants. The PRV gpII, gpIII, and
gp50 products are typical glycoproteins associated with
membranous structures in PRV infected cells. Anti-gpII,
anti-gpIII and anti-gp50 specific monoclonal antibodies
followed by fluorescein-conjugated goat anti-mouse IgG were
used to analyze the PRV glycoprotein expression on the
surface of recombinant infected Vero cells. Surface
expression of neither gpII, gpIII, nor gp50 was detectable
on the surface of mock infected cells or cells infected with
the NYVAC (vP866) parent virus. PRV gpII expression was
observed on the surface of vP881, vP912, vP915, and vP925
infected cells. PRV gpIII surface expression was observed
in vP883, vP915, vP916, and vP925 infected cells. PRV gp50
surface expression was observed in vP900, vP912, vP916, and
vP925 infected cells. In summary, the surface expression of
the particular PRV glycoproteins was only detectable in
cells infected with NYVAC/PRV recombinants containing the
appropriate PRV gene(s).

    Immunoprecipitation of PRV Glycoproteins from Cells
Infected with the NYVAC/PRV Recombinants. The authenticity
of the expressed PRV gpII, gpIII, and gp50 glycoproteins in
Vero cells infected with the NYVAC/PRV recombinants was
analyzed by immunoprecipitation. The PRV gpII gene product
represents one of the major glycoproteins encoded in PRV-
infected cells. The mature protein consists of a complex of
glycoproteins linked by disulfide bonds (Hampl et al., 1984;

Lukacs et al., 1985). Under reducing conditions, three
species are resolved from this complex. These species (IIa-
IIc) migrate with apparent size of 120 kDa, 74-67 kDa, and
58 kDa, respectively, on an SDS-polyacrylamide gel (Hampl et
al., 1984).

In immunoprecipitation analyses using the anti-PRVgpII
specific serum, no PRV-specific protein species were
precipitated from mock infected cells or cells infected with
the NYVAC (vP866) parent virus. PRV gpII was also not
detectable in cells infected with the non-gpII containing
NYVAC/PRV recombinants vP916, vP883, and vP900. It is
evident that PRV gpII was expressed in all the NYVAC/PRV
recombinants which harbor the PRV gpII gene. These are
vP925, vP912, vP915 and vP881. Lysates from Vero cells
infected with the PRV gpII containing recombinants all
contained protein species consistent with the proper
expression and processing of gpII to gpIIa (120 kDa), gpIIb
(74-67 kDa), and gIIc (58 kDa). Two additional protein
species of 45 kDa and 10 kDa were specifically precipitated
with the anti-gpII serum. These protein species appear to
emerge by an aberrant proteolytic processing of PRV gpII at
late times in recombinant infected cells.

The PRV gpIII product is another major PRV
glycoprotein. The gpIII exists as a monomer not complexed
with other viral proteins that migrates with an apparent
molecular weight of 92 kDa (Hampl et al., 1984; Robbins et
al., 1986b). In immunoprecipitation analyses from NYVAC/PRV
recombinant infected cells using antisera specific for
gpIII, no anti-gpIII specific protein species were present
in lysates from mock infected cells, nonrecombinant infected
cells, or cells infected with NYVAC/PRV recombinants not
containing gpIII (vP912, vP881, and vP900, respectively).
Lysates from vP925, vP915, vP916, and vP883 infected cells
all contained the 92 kDa PRV gpIII gene product.

The mature PRV gp50 gene product is approximately 50 to
60 kDa (Petrovskis et al., 1986a; Wathen et al., 1984), that
most likely contains O-linked carbohydrate (Petrovskis et
al., 1986b). In immunoprecipitations from lysates of cells
infected with the NYVAC/PRV recombinants using antisera

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specific to the PRV gp50 gene product, gp50 was not present
in lysates from mock infected cells, nonrecombinant infected
cells, and cells infected with the recombinants not
containing the gp50 gene (vP915, vP881, and vP883,
respectively). Lysates from cells infected with recombinant
NYVAC viruses containing the PRV gp50 gene (vP925, vP912,
vP916, and vP900, respectively) all expressed a 50-60 kDa
protein species which was specifically precipitated with the
anti-PRV gp50 serum.

Table 1. NYVAC Recombinants Expressing PRV glycoproteins
          gpII, gpIII and gp50

Recombinant	Parent	Donor Plasmid	PRV Glycoprotein
vP881	vP866	pPR18	gpII
vP883	vP866	pPRVIIIVCTK	gpIII
vP900	vP866	pATIgp50	gp50
vP912	vP881	pATIgp50	gpII, gp50
vP915	vP883	pPR18	gpII, gpIII
vP916	vP883	pATIgp50	gpIII, gp50
vP925	vP915	pATIgp50	gpII, gpIII, gp50

**Example 11 - CONSTRUCTION OF NYVAC RECOMBINANTS EXPRESSING
               THE gp340, gB and gH GENES OF EPSTEIN-BARR
               VIRUS**

     A NYVAC donor plasmid containing the EBV gp340, gB, and
gH genes was constructed. This donor plasmid was used to
generate two recombinants: vP941 and vP944.

     Restriction enzymes were obtained from Bethesda
Research Laboratories, Inc. (Gaithersburg, MD), New England
BioLabs, Inc. (Beverly, MA) or Boehringer-Mannheim
(Indianapolis, IN). T4 DNA ligase and DNA polymerase I
Klenow fragment were obtained from New England BioLabs, Inc.
Standard recombinant DNA techniques were used (Maniatis et
al., 1982) with minor modifications for cloning, screening
and plasmid purification. Nucleic acid sequences were
confirmed using standard dideoxychain-termination reactions
(Sanger, 1977) on alkaline-denatured double-stranded plasmid
templates. M13mp18 phage, pIBI24 and pIBI25 plasmids were
obtained from International Biotechnologies, Inc., CT.

     Cell Lines and Virus Strains. NYVAC was used as a
rescue virus to generate recombinants. All vaccinia virus

stocks were produced in Vero (ATCC CCL81) cells in Eagles
MEM medium supplemented with 5-10% newborn calf serum (Flow
Laboratories, Mclean, VI).

Oligonucleotide-Directed Mutagenesis. The uracil-
substituted single-stranded DNA template used for the
mutagenesis reactions was from CJ236 transformed cells. The
mutations were achieved by using the protocol of Kunkel et
al. (1987). The various oligonucleotides were synthesized
using standard chemistries (Biosearch 8700, San Rafael, CA;
Applied Biosystems 380B, Foster City, CA)

Construction of Vaccinia Virus Recombinants.
Procedures for transfection of recombinant donor plasmids
into tissue culture cells infected with a rescuing vaccinia
virus and identification of recombinants by in situ
hybridization on nitrocellulose filters were as previously
described (Panicali et al., 1982; Piccini et al., 1987).

Modifications and Expression in Vaccinia Recombinants
of EBV Genes gp340, gB, and gH. The gp340 gene corresponds
to the open reading frame BLLF1a of the complete EBV
sequence (Baer et al., 1984). The gp220 gene derives from
the gp340 mRNA by an internal splicing event (open reading
frame BLLF1b). The gp340 and gp220 genes were isolated from
cDNA clones (plasmids pMLPgp340 and pMLPgp220, respectively)
provided by Dr. Perricaudet (Centre de Recherche sur le
Cancer-IRSG, 7 rue Guy Mocquet, 94802 Villejuif, France).

The 2100 bp XmaI/ClaI fragment of pMLPgp220 was
inserted into XmaI/ClaI M13 mp18, and the resulting plasmid
was called mp18gp220. By in vitro mutagenesis using the
oligonucleotides CM4 and CM5 the 5' and 3' extremities of
gp220 gene were modified for expression under the control of
the vaccinia H6 promoter. The plasmid containing the
modified gp220 gene was called mp18gp220(4+5). The
nucleotide composition of CM4 (SEQ ID NO:60) and CM5 (SEQ ID
NO:61) were as follows:

CM4: TAAAGTCAATAAATTTTTATTGCGGCCGCTACCGAGCTCGAATTCG
                                 NotI

CM5: GCTTGCATGCCTGCAGATATCCGTTAAGTTTGTATCGTAATGGAGGCAGCCTTGC
                        EcoRV   Met

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The 2300 bp NarI/EcoRV fragment of mp18gp220(4+5) was
cloned into the NarI/EcoRV plasmid SP131NotI. SP131NotI
contains the complete H6 vaccinia promoter as previously
defined (Taylor et al., 1988a, b). The resulting plasmid
was called SP131gp220.

The 2360 bp ScaI/XhoI fragment of pMLPgp340 was cloned
into the ScaI/XhoI SP131gp220 plasmid. The resulting
plasmid was called SP131gp340.

The 2800 bp NotI/NotI fragment of SP131gp340 was cloned
into the SmaI digested vaccinia donor plasmid pSD486. The
resulting plasmid was called 486H6340.

The EBV gB gene corresponds to the open reading frame
BALF4 of the complete EBV sequence (Baer et al., 1984). A
3500 bp EcoRI/XmnI fragment was isolated from the EBV BamHI
A fragment and cloned into the HincII/EcoRI plasmid pIBI25.
The resulting plasmid was called p25gB.

By in vitro mutagenesis, using the oligonucleotides
EBVM5 (SEQ ID NO:62) and EBVM3 (SEQ ID NO:63), the EBV gB
gene was adapted for expression under the control of the
vaccinia H6 promoter. The nucleotide composition of EBVM5
(SEQ ID NO:62) and EBVM3 (SEQ ID NO:63) were as follows:
EBVM5:
CCCTACGCCGAGTCATTACGATACAAACTTAACGGATATCAGAGTCGTACGTAGG
EBVM3: CTGGAAACACTTGGGAATTCAAGCTTCATAAAAAGGGTTATAGAAGAGTCC
The resulting plasmid was called p25gB(5+3).

The 2600 bp EcoRV/EcoRI fragment of p25gB(5+3) was
cloned into the EcoRV/EcoRI Sp131 plasmid. The resulting
plasmid was called SP131gB.

The EBV gH gene corresponds to the BXLF2 open reading
frame of the complete EBV sequence (Baer et al., 1984). The
complete BXLF2 open reading frame is contained in two BamHI
EBV fragments: BamHI X and BamHI T. The complete BXLF2 open
reading frame was reconstituted by cloning the 830 bp
SmaI/BamHI fragment of EBV BamHI T fragment into the
SmaI/BamHI pIBI24 plasmid; the resulting plasmid was called
24gH5. The 1850 bp BamHI/HindIII fragment of EBV BamHI X
fragment was cloned into the BamHI/HindIII 24gH5 plasmid.
The resulting plasmid was called 24gH.

By *in vitro* mutagenesis using the oligonucleotides HM5, HM4, and HM3 the EBV gH gene was modified to be expressed under the control of the vaccinia B13R hemorrhagic promoter (Goebel et al., 1990a,b). The oligonucleotide HM4 was used to modify a sequence corresponding to a vaccinia early transcription termination signal. The nucleotide compositions of HM5 (SEQ ID NO:64), HM4 (SEQ ID NO:65), and HM3 (SEQ ID NO:66) were as follows:

HM5: ACACAGAGCAACTGCAGATCTCCCGATTTCCCCTCT

HM4: GGGCAAAGCCACAAAATATGCAGGATTTCTGCG

HM3: GCCAGGGTTTTCCCAGATCTGATAAAAACGACGGCCAGTG

The resulting plasmid containing the modified gH was called 24gH(5+4+3).

The vaccinia hemorrhagic promoter does not appear to be a strong promoter when compared with other pox promoters. The EBV gH gene has been placed under the control of the 42 kDa entomopox promoter. This was achieved by using the polymerase chain reaction (PCR), specific oligonucleotides 42gH (SEQ ID NO:67) and BAMgH (SEQ ID NO:68) and the plasmid 24gH(5+4+3) as template.

42gH: GGGTCAAAATTGAAAATATATAATTACAATATAAAATGCAGTTGCTCTGTGTT

                                        Met

BAMgH: ATGGATCCTTCAGAGACAG   (The first A residue
                              corresponds to position 292 of
                              the gH coding sequence)

The PCR reaction was processed in a Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) with 36 cycles at 94°C for 1 minute, 42°C for 1.5 minutes, and 72°C for 3 minutes, and a final extension step at 72°C for 5 minutes. The PCR product was purified, digested with BamHI and cloned into the 4550 bp SmaI/BamHI fragment of 24gH(5+4+3). The resulting plasmid was called 24BXLF2.42K.

Insertion of EBV gp340, gB, and gH Genes into the Vaccinia Donor Plasmid pSD542 and Isolation of vP941 and vP944. The vaccinia donor plasmid pSD542 is a derivative of pSD460 with an expanded polylinker region; it is used to recombine foreign genes into the vaccinia TK locus.

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The 2820 bp BamHI/BglII fragment of 486H6340 plasmid
was cloned into the BamHI/BglII pSD542 plasmid. The
resulting plasmid was called 542.340.

The 2150 bp SmaI/BglII fragment of 24BXLF2.42K plasmid
was cloned into the SmaI/BglII 542.340 plasmid. The
resulting plasmid was called 542.340gH.

The 2700 bp HindIII/HindIII fragment of SP131gB plasmid
was cloned into the BglII 542.340gH plasmid. The resulting
plasmid was called EBV Triple.1. A map of the EBV coding
regions inserted into EBV Triple.1 plasmid is presented in
FIG. 8. The direction of transcription is indicated by the
arrows in FIG. 8.

EBV Triple.1 plasmid was digested by NotI and
transfected into Vero cells infected with NYVAC or vP919, a
NYVAC based vaccinia recombinant containing three HBV genes.
The corresponding recombinant vaccinia viruses vP944 and
vP941 were isolated.

**Example 12 - CONSTRUCTION OF NYVAC RECOMBINANTS EXPRESSING
                THE gB, gC and gD GENES OF HERPES SIMPLEX
                VIRUS TYPE 2**

A recombinant vaccinia virus that expresses the HSV2
gB, gC and gD genes was constructed.

Cells and Viruses. HSV 2 (strain G) was propagated in
VERO cells (ATCC CCL81) and purified by centrifugation on a
sucrose gradient (Powell et al., 1975).

Vaccinia virus (Copenhagen) and recombinants derived
therefrom were propagated in VERO cells (ATCC CCL81) as
previously described (Panicali et al., 1982; Guo et al.,
1989).

Isolation of the HSV2 gB Gene. A 12 kb BglII fragment,
containing the HSV2 gB gene, was isolated from HSV2 genomic
DNA and inserted into the BamHI site ofsite of pSD48 pUC19.
The resulting plasmid was designated pJ4.

The gB gene was then cloned between vaccinia virus
flanking arms. This was accomplished by cloning the 2,700
bp SstII-SacI (partial) fragment of pJ4 into the SstII-SacI
fragment of pMP409DVC (Guo et al., 1989). This placed the
gB gene between the vaccinia virus sequences flanking the
M2L gene. The plasmid generated by this manipulation was
designated pGB1.

An in-frame termination codon was then added to the 3'-
end of the gB gene. This was accomplished by cloning the
oligonucleotides, GBL3 (SEQ ID NO:69) 5'-CTAATAG-3' and GBL4
(SEQ ID NO:70) 5'-GATCCTATTAGAGCT-3', into the 6,300 bp
BamHI-SacI (partial) fragment of pGB1. The plasmid
generated by this manipulation was designated pGB2.

The vaccinia virus H6 promoter (Taylor et al., 1988a,
b; Perkus et al., 1989) was then cloned upstream of the gB
gene. This was accomplished by cloning the 370 bp BglII
fragment of pBLVH14 (Portetelle et al., 1991), containing
the H6 promoter, into the BglII site of pGB2. The plasmid
generated by this manipulation was designated pGB3.

The initiation codon of the H6 promoter was then
aligned with the initiation codon of the gB gene. This was
accomplished by cloning the oligonucleotides, GBL1 (SEQ ID
NO:71) 5'-
ATCCGTTAAGTTTGTATCGTAATGCGCGGGGGGGGCTTGATTTGCGCGCTGGTCGTGGGG
GCGCTGGTGGCCGC-3' and GBL2 (SEQ ID NO:72) 5'-
GGCCACCAGCGCCCCACGACCAGCGCGCAAATCAAGCCCCCCCCGCGCATTACGATACA
AACTTAACGGAT-3', into the 6,300 bp SstII-EcoRV (partial)
fragment of pGB3. The plasmid generated by this
manipulation was designated pGB5.

The H6-promoted gB gene was then cloned into a
different vaccinia virus donor plasmid. This was
accomplished by cloning the 2,800 bp BglII-BamHI fragment of
pGB5, containing the H6- promoted gB gene, into the BglII
site of pSD513VCVQ. (pSD513VCVQ is a subclone of the
vaccinia virus HindIII J fragment in which the thymidine
kinase (tk) gene is replaced by a polylinker region.) This
placed the H6-promoted gB gene between the vaccinia virus
sequences flanking the tk gene. The plasmid generated by
this manipulation was designated pGB6.

Isolation of the HSV2 gC Gene. A 2,900 bp SalI
fragment, containing the HSV2 gC gene, was isolated from
HSV2 genomic DNA and inserted into the SalI site of pIBI25.
The resulting plasmid was designated pGC3.

The gC gene was then cloned between vaccinia virus
flanking arms. This was accomplished by cloning the 2,900
bp XhoI-BamHI fragment of pGC3 into the XhoI-BamHI site of

pGC2. pGC2 was generated by cloning the 370 bp BglII
fragment of pBLVH14 (Portetelle et al., 1991), containing
the H6 promoter, into the BglII site of pSD486 (FIG. 2).
This placed the gC gene between the vaccinia virus sequences
flanking the u gene. The plasmid generated by this
manipulation was designated pGC5.

     The initiation codon of the H6 promoter was then
aligned with the initiation codon of the gC gene. This was
accomplished by cloning the oligonucleotides, GCL1 (SEQ ID
NO:73) 5'-
ATCCGTTAAGTTTGTATCGTAATGGCCCTTGGACGGGTGGGCCTAGCCGTGGGCCTGTG-
3' and GCL2 (SEQ ID NO:74) 5'-
AGGCCCACGGCTAGGCCCACCCGTCCAAGGGCCATTACGATACAAACTTAACGGAT-3',
into the 5,400 bp NruI-SfiI fragment of pGC5. The plasmid
generated by this manipulation was designated pGC10.

     Extraneous 3'-noncoding sequence was then eliminated
from pGC10. This was accomplished by recircularizing the E.
coli DNA polymerase I (Klenow fragment) filled-in 4,900 bp
SalI-SmaI (partial) fragment of pGC10. The plasmid
generated by this manipulation was designated pGC11.

     Additional 3'-noncoding sequence was then eliminated
from pGC11. This was accomplished by cloning the
oligonucleotide, GCL3 5'-CTAGGGGCC-3', into the 4,900 bp
XbaI-ApaI (partial) fragment of pGC11. The plasmid
generated by this manipulation was designated pGC12.

     Isolation of the HSV2 gD Gene. A 7.5 kb XbaI fragment,
containing the HSV2 gD gene, was isolated from HSV2 genomic
DNA and inserted into the XbaI site of pIBI25. The
resulting plasmid was designated pGD1.

     The gD gene was then cloned downstream of the H6
promoter and between vaccinia virus flanking arms. This was
accomplished by cloning the 1,500 bp DraI-PstI fragment of
pGD1 into the 3,700 bp SmaI-PstI fragment of pTP15 (Guo et
al., 1989). This placed the gD gene downstream of the H6
promoter and between the vaccinia virus sequences flanking
the HA gene. The plasmid generated by this manipulation was
designated pGD2.

     The initiation codon of the H6 promoter was then
aligned with the initiation codon of the gD gene. This was

accomplished by cloning the oligonucleotides, GDL1 (SEQ ID
NO:75) 5'-ATCCGTTAAGTTTGTATCGTAATGGGGCGTTTGACCTCCGG-3' and
GDL2 (SEQ ID NO:76) 5'-
CGCCGGAGGTCAAACGCCCCATTACGATACAAACTTAACGGAT-3', into the
5,100 bp EcoRV-AhaII (partial) fragment of pGD2. The
plasmid generated by this manipulation was designated pGD5.

Extraneous 3'-noncoding sequence was then eliminated.
This was accomplished by cloning the oligonucleotides, GDL3
(SEQ ID NO:77) 5'-
GGCAGTACCCTGGCGGCGCTGGTCATCGGCGGTATTGCGTTTTGGGTACGCCGCCGGCGC
TCAGTGGCCCCCAAGCGCCTACGTCTCCCCCACATCCGGGATGACGACGCGCCCCCCTCG
CACCAGCCATTGTTTTACTAGCTGCA-3' and GDL4 (SEQ ID NO:78) 5'-
GCTAGTAAAACAATGGCTGGTGCGAGGGGGGCGCGTCGTCATCCCGGATGTGGGGGAGAC
GTAGGCGCTTGGGGGCCACTGAGCGCCGGCGGCGTACCCAAAACGCAATACCGCCGATGA
CCAGCGCCGCCAGGGTACTGCC-3', into the 4,800 bp NaeI-PstI
fragment of pGD5. The plasmid generated by this
manipulation was designated pGD7.

Additional sequence was then added upstream of the H6
promoter. This was accomplished by cloning the 150 bp
BglII-EcoRV fragment of pGB6 (see above) into the 4,800 bp
BglII-EcoRV fragment of pGD7. The plasmid generated by this
manipulation was designated pGD8.

Construction of a Vaccinia Virus Donor Plasmid
Containing the HSV2 gB, gC and gD Genes. A plasmid
containing the gC and gD genes was constructed. This was
accomplished by cloning the 1,850 bp PstI fragment of pGC12,
containing the H6-promoted gC gene, into the PstI site of
pGD8. The plasmid generated by this manipulation was
designated pGCD1.

A plasmid containing the gB, gC and gD genes was then
constructed. This was accomplished by cloning the 2,800 bp
BglII-BamHI fragment of pGB6, containing the H6-promoted gB
gene, into the 6,800 bp BamHI (partial) fragment of pGCD1.
The plasmid generated by this manipulation was designated
pGBCD1.

Extraneous DNA was then eliminated. This was
accomplished by cloning the E. coli DNA polymerase I (Klenow
fragment) filled-in 6,000 bp HindIII-BamHI (partial)
fragment of pGBCD1, containing the H6-promoted gB, gC and gD

genes, into the SmaI site of pMP831. The plasmid generated
by this manipulation was designated pGBCDC1.

The H6-promoted gB, gC and gD genes were then cloned
between vaccinia virus flanking arms. This was accomplished
by cloning the oligonucleotides, HSVL1 (SEQ ID NO:79) 5'-
TCGATCTAGA-3' and HSVL2 (SEQ ID NO:80) 5'-AGCTTCTAGA-3', and
the 5,700 bp HindIII-BamHI (partial) fragment of pGBCDC1,
containing the H6-promoted gB, gC and gD genes, into the
3,600 bp XhoI-BglII fragment of pSD541. This placed the H6-
promoted gB, gC and gD genes between the vaccinia virus
sequences flanking the ATI gene. The plasmid generated by
this manipulation was designated pGBCD4.

Construction of vP914. A vaccinia virus recombinant,
vP914, containing the HSV2 gB, gC and gD genes, was
constructed. The procedures used to construct vaccinia
virus recombinants have been described previously (Panicali
et al., 1982; Guo et al., 1989; Guo et al., 1990). The
vaccinia virus recombinant, vP914, was generated by
transfecting pGBCD4 into vP866 (NYVAC) infected cells. The
HSV2 genes in this recombinant are under the transcriptional
control of the vaccinia virus H6 promoter.

Immunofluorescence and Immunoprecipitation of vP914
Infected Cells. Immunofluorescence and immunoprecipitations
were performed as previously described (Guo et al., 1989).
Rabbit antisera against HSV2 was obtained from DAKO Corp.
(code no. B116). Monoclonal antibodies against HSV2 gB
(H233) and HSV2 gD (HD1) (Meignier et al., 1987) were
obtained from B. Meignier (Institut Merieux, Lyon, France).

In HSV2 infected cells, gB, gC and gD (as well as other
HSV2 glycoproteins) are expressed on the cell surface.
Immunofluorescence studies with vP914 infected cells, using
monoclonal antibodies specific for HSV2 gB (H233) and HSV2
gD (HD1), indicated that the HSV2 gB and gD glycoproteins
produced in these cells were also expressed on the cell
surface.

In HSV2 infected cells, gB, gC and gD have molecular
weights of approximately 117 kDa, 63 kDa and 51 kDa,
respectively (Marsden et al., 1978; Marsden et al., 1984;
Zweig et al., 1983). Immunoprecipitation of vP914 infected

cells with a gB-specific monoclonal antibody (H233)
precipitated three major proteins with molecular weights of
approximately 117 kDa, 110 kDa and 100 kDa, as well as other
minor proteins. Immunoprecipitation with a gD-specific
monoclonal antibody (HD1) precipitated a major protein with
a molecular weight of approximately 51 kDa and minor
proteins with molecular weights of approximately 55 kDa and
46 kDa. Additionally, immunoprecipitation of vP914 infected
cells with polyclonal antisera against HSV2 precipitated a
protein with a molecular weight similar to gC, 63 kDa, (as
well as an 85 kDa protein) and proteins corresponding in
size to gB and gD. Therefore, cells infected with vP914
appeared to express HSV2 proteins with molecular weights
similar to gB, gC and gD.

**Example 13 - CONSTRUCTION OF NYVAC RECOMBINANTS EXPRESSING
HEPATITIS B VIRUS GENES**

DNA Cloning and Synthesis. Plasmids were constructed,
screened and grown by standard procedures (Maniatis et al.,
1982; Perkus et al., 1985; Piccini et al., 1987).
Restriction endonucleases were obtained from Bethesda
Research Laboratories (Gaithersburg, MD), New England
Biolabs (Beverly, MA) and Boehringer Mannheim Biochemicals
(Indianapolis, IN). T4 DNA ligase was obtained from New
England Biolabs. T4 polynucleotide kinase was obtained from
Bethesda Research Laboratories. Plasmid pGEM-3Z was
obtained from Promega (Madison, WI). The origin of plasmid
pTHBV containing the HBV genome cloned in pBR322 has been
previously described (Paoletti et al., 1984).

Synthetic oligodeoxyribonucleotides were prepared on a
Biosearch 8750 or Applied Biosystems 380B DNA synthesizer as
previously described (Perkus et al., 1989). DNA sequencing
was performed by the dideoxy-chain terminating method
(Sanger et al., 1977) using Sequenase (Tabor and Richardson,
1987) as previously described (Guo et al., 1989). DNA
amplification by polymerase chain reaction (PCR) for cloning
and sequence verification (Engelke et al., 1988) was
performed using custom synthesized oligonucleotide primers
and GeneAmp DNA amplification Reagent Kit (Perkin Elmer

Cetus, Norwalk, CT) in an automated Perkin Elmer Cetus DNA
Thermal Cycler.

Virus and Transfection. The NYVAC strain of vaccinia
virus and its intermediate ancestor, vP804 (FIG. 5), were
used. Generation and processing of recombinant virus are as
previously described (Panicali et al., 1982).

Immunoprecipitation. Vero cells were infected at an
m.o.i. of 10 pfu per cell with recombinant vaccinia virus,
with the NYVAC parent virus (vP866) or were mock infected.
After a 1 hour adsorption period, the inoculum was removed
and infected cells were overlayed with methionine-free media
containing ^{35}S-methionine (20 uCi/ml). All samples were
harvested at 8 hours post infection. Samples were lysed in
3x buffer A containing triton and DOC (3% NP-40, 3% triton,
3% DOC, 30 mM Tris pH 7.4, 450 mM NaCl, 3 mM EDTA, 0.03 %
NaAzide, 0.6 mg/ml PMSF) containing 50 ul aprotinin (Sigma
Chemical Co., St. Louis, MO, # A6279). All lysates were
precleared against normal rabbit sera linked to protein A-
sepharose.

Rabbit antisera raised to HBV core antigen and to HBV
S2 peptide (aa 120-153) were obtained from R. Neurath (The
Lindsley F. Kimball Research Institute of the New York Blood
Center). Anti-S2 antiserum was preadsorbed with vP866
infected Vero cells. HBV proteins were immunoprecipitated
using anti-core or anti-S2 antiserum and resuspended in 2x
Laemmli sample buffer (Laemmli, 1970) for electrophoresis
and subsequent autoradiography.

Serology. Rabbits and guinea pigs were inoculated with
10^8 pfu recombinant vaccinia virus vP919 in sets of two by
intradermal, subcutaneous or intramuscular route. Six weeks
after the primary inoculation, rabbits were boosted once by
the same route and dose. Seven weeks after the primary
inoculation, guinea pigs were boosted once by the same route
and dose. Groups of 12 mice were inoculated with 10^7 pfu
recombinant vaccinia virus vP919 by intradermal,
subcutaneous or intramuscular route. Seven weeks after the
primary inoculation, mice were boosted once by the same
route. Sera were collected at weekly intervals. Weekly
bleedings from each group of mice were pooled. All sera

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were analyzed for antibody to HBV surface antigen using the
AUSAB radioimmunoassay kit (Abbott, North Chicago, IL). All
sera were analyzed for antibody to HBV core antigen using
the CORAB competitive radioimmunoassay kit (Abbott) using
standard techniques.

   Construction of vP919. Vaccinia recombinant vP919
contains three genes from Hepatitis B Virus inserted into
NYVAC vaccinia virus vector. The genes were inserted
individually into three different sites of the virus. The
three HBV genes encode the following protein products: (1)
HBV M protein, (referred to here as small pre S antigen, or
spsAg), (2) HBV L protein (referred to here as large pre S
antigen, or lpsAg) and (3) a fusion protein, (referred to
here as S12/core) composed of the entire pre-S region (S1 +
S2) linked onto the amino terminus of the core antigen.

   Vaccinia virus does not maintain multiple copies of the
same heterologous DNA sequences inserted contiguously into a
single vaccinia genome (Panicali et al., 1982) Since coding
sequences for the spsAg are contained within coding
sequences for the lpsAg, insertion of both genes into a
single vaccinia genome would be expected to lead to
instability of the genome. Similarly, an S1+S2 DNA region
present in a hybrid S12/core gene could undergo
recombination with the equivalent S1+S2 region of lpsAg.
These potential problems were prevented in two ways. (1)
The three genes were inserted into three different loci in
the vaccinia genome, separated from each other by large
regions of vaccinia DNA containing essential genes. Thus,
any recombination between the HBV genes would lead to
incomplete vaccinia genomes which would not produce viable
vaccinia progeny. (2) DNAs encoding the spsAg gene and the
S1+S2 region of the S12/core hybrid gene were synthesized
chemically with different codon usage to minimize DNA
homology with the native HBV gene encoding the lpsAg and
with each other. The native HBV gene encoding the lpsAg and
the synthetic gene encoding the spsAg are of the ayw
subtype; the S1+S2 region for the fusion S12/core gene was
synthesized to correspond to the ad₁ subtype (Valenzuela et
al., 1979).

Cassettes containing the three individual HBV genes
under the control of poxvirus promoters were assembled in
different vaccinia donor plasmids and inserted sequentially
into vaccinia virus as detailed below.

The synthetic version of the gene encoding the HBV
spsAg was synthesized using vaccinia favored codons with the
following deviations. (1) The T5NT early transcription
terminator TTTTTCT occurring in amino acids 19 through 21 of
the sAg (HBV S protein) was modified to TTCTTTC, and codon
utilization was adjusted to prevent the generation of other
T5NT termination signals (Yuen et al., 1987). (2) To avoid
possible aberrant translation products, codon usage was
adjusted to prevent the generation of any out of frame ATG
initiation codons in either direction. The synthetic spsAg
gene was linked precisely to the modified synthetic vaccinia
virus H6 early/late promoter (Perkus et al., 1989). The
complete sequence of promoter and gene is given in FIG. 9.
Amino acid sequence is based on the sequence in plasmid
pTHBV, which differs from the published ayw sequence
(Galibert et al., 1979) at two amino acid positions in the
S2 region: Galibert, aa 31 thr; aa 36 leu; pTHBV, aa 31 ala;
aa 36 pro.

Plasmid pGJ15 contains the H6 promoter/synthetic spsAg
gene in the vaccinia ATI insertion locus (Perkus et al.,
1990). pGJ15 was constructed by assembling portions of the
synthetic spsAg gene in pGEM-3Z, then transferring the
assembled gene to insertion plasmid pMP494H, a derivative of
pSD492 which contains the synthetic H6 promoter in the ATI
deletion locus.

Referring now to FIG. 10, the synthetic HBV spsAg was
assembled in three parts. Plasmids pGJ5, pGJ3, and pGJ7
were generated from 6, 5, and 8 pairs of complementary
oligonucleotides respectively as follows. Complementary
oligonucleotide pairs synthesized with standard chemistries
were kinased under standard conditions followed by heating
at 65°C and allowed to cool slowly to room temperature to
effect annealing. Aliquots of the annealed pairs comprising
each fragment were combined with appropriately digested
pGEM-3Z (Promega) and ligated under standard conditions.

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Fragment SX (indicated with a solid box), bounded by SphI
and XbaI restriction sites, was ligated to pGEM-3Z vector
plasmid digested with those enzymes creating plasmid pGJ5.
Vector plasmid sequences are indicated with open regions.
Similarly, fragments XB (diagonal cross-hatch) and BH
(horizontal cross-hatch), were assembled in plasmid pGEM-3Z
digested with either XbaI and BamHI, or BamHI and HindIII,
respectively, generating plasmids pGJ3 and pGJ7. The
integrity of the insert in each plasmid was verified by
determination of the DNA sequence.

Synthetic HBV gene fragments were isolated by digestion
of the plasmids pGJ5, pGJ3 and pGJ7 with the appropriate
restriction enzymes flanking the SX, XB and BH gene segments
and subsequently ligated to pGEM-3Z digested with SphI and
HindIII generating plasmid pGJ9 which contains the
contiguous HBV synthetic spsAg sequence. Oligonucleotides
H6LINK (SEQ ID NO:81)
(5'-CTCGCGATATCCGTTAAGTTTGTATCGTAATGCAGTGG-3') and H6LINK2
(SEQ ID NO:82)
(5'-AATTCCACTGCATTACGATACAAACTTAACGGATATCGCGAGGTAC-3')
containing the 3' 28 bp of the H6 promoter (diagonal hatch)
appended to the synthetic spsAg at the initiating methionine
through the EcoRI site 9 bp downstream from the first codon,
were ligated to pGJ9 digested with KpnI (5' to the SphI site
within the multiple cloning region derived from pGEM-3Z) and
with EcoRI, generating plasmid pGJ12. A NruI/HpaI fragment
was isolated from pGJ12 and ligated to similarly digested
pMP494H, generating plasmid pGJ15. pMP494H is an ATI
insertion plasmid containing the vaccinia H6 promoter in the
ATI deletion region. pGJ15 contains the H6 promoter-driven
HBV synthetic spsAg gene flanked by vaccinia sequences
(stippled) surrounding the ATI locus.

pGJ15 was used as donor plasmid for recombination with
vaccinia recombinant vP804, generating recombinant vaccinia
virus vP856. vP804 contains the NYVAC deletions for the TK,
HA, u and [C7L - K1l]. Recombinant virus vP856 contains the
above deletions with the insertion of the HBV synthetic
spsAg gene replacing the ATI region. Progeny virus
recombinant described below containing an insert in the I4L

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region will be equivalent to NYVAC in terms of deletions
(TK, HA, ATI, I4L, u, [C7L - K1L]).

The gene encoding the HBV lpsAg was derived from
plasmid pTHBV. In addition to the amino acid changes in the
S2 region referred to above, pTHBV differs from the
published ayw subtype at one amino acid position in the S1
region: Galibert et al., 1979, aa 90 ser; pTHBV aa 90 thr.
The early translational termination signal in sAg referred
to above was modified from TTTTTCT to TTCTTCT. The entire
lpsAg gene was placed under the control of the 105 bp cowpox
u promoter (Pickup et al., 1986)   The entire sequence of
the u promoter/lpsAg gene cassette is given in FIG. 11.

Plasmid pMP550ulps contains the u promoter/lpsAg gene
in the vaccinia I4L deletion locus. The construction of
pMP550ulps is presented schematically in FIG. 12. The I4L
deletion in pMP550ulps is equivalent to the I4L deletion in
NYVAC.

Referring now to FIG. 12(A), by PCR using synthetic
oligonucleotide primers MPSYN322 (SEQ ID NO:83), MPSYN323
(SEQ ID NO:84) and template plasmid pBScow, the 5' end of
the HBV lpsAg gene was added to the cowpox u promoter
(orientation indicated by an arrow) generating pMPuS1. (The
dark box indicates the u promoter and the striped box
indicates HBV sequences.) pSD550 is a vaccinia insertion
plasmid for the I4L deletion region. (The triangle
indicates the site of deletion and the open box indicates
vaccinia sequences.) A SnaBI/BamHI fragment containing the
u promoter/HBV junction was isolated and inserted into
pSD550 cut with SmaI/BamHI, forming pMP550u.

Referring now to FIG. 12(B), a 1.1 kb DraI fragment
containing the entire HBV lpsAg was isolated from pTHBV and
inserted into pUC8, generating pMP8S. Translation
initiating codon and stop codon are indicated. (*)
indicates the site of T5NT transcriptional termination
signal (Yuen et al., 1987). The transcriptional termination
signal was removed from pMP8S by PCR mutagenesis as
indicated, generating pMP8ST. A 1.1 kb BamHI (partial)
fragment containing the bulk of the lpsAg gene was isolated
from pMP8ST and inserted into plasmid pMP550u cut with

BamHI, generating pMP550ulps. pMP550ulps was used for
recombination with vaccinia recombinant vP856, generating
vP896. Synthetic oligonucleotide sequences are as follows:

                              BglII ClaI
MPSYN322 (SEQ ID NO:83) 5' CCCAGATCTATCGATTGCCATGGGGCAGA 3'

                                 BamHI
MPSYN323 (SEQ ID NO:84) 5' TCTGAAGGCTGGATCCAACT 3'

                                 XhoI
MPSYN330 (SEQ ID NO:85) 5' CAATCTTCTCGAGGATT 3'

                             HincII
MPSYN331 (SEQ ID NO:86) 5' AACAAGAAGAACCCCGCC 3'

The HBV initiation codon in MPSYN322 (SEQ ID NO:83) is
underlined, the mutated base in MPSYN331 (SEQ ID NO:86) is
underlined and restriction sites are indicated.

     pMP550ulps was used as donor plasmid for recombination
with rescuing virus vP856 described above to generate
recombinant virus vP896. vP896 contains both the genes for
HBV spsAg and HBV lpsAg in a NYVAC background (deletion of
TK, HA, ATI, I4L, u, [C7L - K1L]). To generate a
recombinant containing only the HBV lpsAg gene for purposes
of comparison with multivalent HBV vaccinia recombinants,
pMP550ulps was also used in recombination with vP866
(NYVAC), generating recombinant virus vP897.

     The third HBV gene inserted into vaccinia virus encodes
a fusion protein. Synthetic DNA specifying the HBV S1 and
S2 regions was cloned onto the 5' end of the gene specifying
the HBV core antigen. Synthetic DNA was designed to encode
the S1 + S2 regions of the adw subtype (Valenzuela et al.,
1979), starting with the met at aa position 12 (equivalent
to position 1 of the ayw subtype) (Galibert et al., 1979).
Total translation region of S1 + S2 is 163 codons. To
prevent unwanted intramolecular recombination among HBV
genes in a multivalent HBV vaccinia recombinant virus, codon
utilization was adjusted to minimize DNA homology of the
synthetic S1 + S2 region with the native ayw S1 + S2 region
present in pTHBV and as well as the synthetic S2 region in
pGJ15.

     The entire gene encoding the core antigen was obtained
from pTHBV. The amino acid sequence of the core antigen

encoded by pTHBV agrees with the published ayw sequence
(Galibert et al., 1979). The hepatitis fusion gene encoding
S12/core was placed under the control of the vaccinia I3L
early/intermediate promoter (Vos et al., 1988; Goebel et
al., 1990b positions 64,973 - 65,074). The entire sequence
of the I3L promoter/S12/core gene cassette is given in FIG.
13 (SEQ ID NO:87).

     Plasmid pMP544I3S12C contains the I3L
promoter/S1+S2/core gene in the HA deletion locus (Guo et
al., 1989). The construction of pMP544I3S12C is presented
schematically in FIG. 14.

     Referring now to FIG. 14, plasmid pMPCA-B contains a 1
kb HhaI fragment from pTHBV inserted into the SmaI site of
pUC9. pMP9CA-B contains the entire coding sequences for the
HBV core antigen, as well as flanking HBV DNA upstream and
downstream from the gene. pMP9CA-B was cut with BglII
(partial) 30 bp upstream from the 3' end of the gene and
with EcoRI in the polylinker region at the HBV/pUC junction.
The 3.4 kb vector fragment containing the bulk of the HBV
gene was isolated and ligated with annealed synthetic
oligonucleotides MPSYN275/MPSYN276, (SEQ ID NO:88/SEQ ID
NO:89)

                          BglII
MPSYN275 (SEQ ID NO:88) 5'GATCTCAATCTCGGGAATCTCAATGTTAGAT-

                                 SmaI
                        AACTAATTTTTATCCCGGGT 3'

MPSYN276 (SEQ ID NO:89) 3' AGTTAGAGCCCTTAGAGTTACA-

                        ATCTATTGATTAAAAATAGGGCCCATTAA 5'

generating pMP9CA-C. Restriction sites are indicated, the
translational stop codon is underlined and the early
vaccinia transcriptional terminator is overlined.

     pMP9CA-C contains the entire coding sequence for the
HBV core antigen, and was used as the source for the bulk of
the gene as indicated above.

     The synthetic S1+S2 region was assembled in five double
stranded sections A through E as indicated above using
synthetic oligonucleotides, MPSYN290 through MPSYN308 (SEQ
ID NO:90)-(SEQ ID NO:99), as set out below.
Oligonucleotides ranged in size from 46mer through 71mer,

with 4 to 8 bp sticky ends. 5' ends of oligonucleotides
which were at internal positions within a section were
kinased before annealing of the section. Sequence of
synthetic oligonucleotides used to construct sections A
through E are given below. Only the coding strand is shown.
Relevant restriction sites are noted. Initiation codons for
S1 (section A), S2 (section C) and core (section E) are
underlined.

Section A, MPSYN290-294 (SEQ ID NO:90)-(SEQ ID NO:92)

<pre>
                         HindIII RsaI   (I3L)   (S1)
MPSYN290 (SEQ ID NO:90) 5'AGCTTGTACAATTATTTAGGTTTAATCATGGGAA

                                         CGAACCTATCTGTT 3'

MPSYN292 (SEQ ID NO:91) 5'CCCAACCCACTTGGATTTTTTCCTGATCATCAGT

                                         TAGACCCTGCTTTC 3'

MPSYN294 (SEQ ID NO:92) 5'GGAGCCAACTCAAACAATCCTGACTGGGATTT

                                                      PstI
                           TAACCCCGTCAAAGACGATTGGCCTGCA 3'
</pre>

Section B, MPSYN296-299

<pre>
                        PstI
MPSYN296 (SEQ ID NO:93) 5'GCCAACCAAGTAGGTGTGGGAGCTTTCGGACC-

                          AAGGCTCACTCCTCCACACGGCGGT 3'

MPSYN298 (SEQ ID NO:94) 5'ATATTAGGTTGGTCTCCACAAGCTCAAGG-

                                           HincII EcoRI
                          CATATTGACCACAGTGTCAACCCG 3'
</pre>

Section C, MPSYN300-303

<pre>
                        HindIII HincII
MPSYN300 (SEQ ID NO:95) 5' AGCTTGTCAACAATTCCTCCACCAGCCTCT-

                                    ACTAATCGGCAGTCTGGT 3'

MPSYN302 (SEQ ID NO:96) 5' AGACAGCCAACTCCCATCTCTCCTCCTCTA-

                                         (S2)   EcoRI
                          AGAGACAGTCACCCACAAGCTATGCAGTGG 3'
</pre>

Section D, MPSYN304-305

<pre>
                        HindIII EcoRI
MPSYN304 (SEQ ID NO:97) 5' AGCTTGGGAATTCAACTGCTTTTCACCAG-
</pre>

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                 ACACTTCAAGACCCTAGAGTCAGGGGTCTATATCTTCCTGCA 3'

Section E, MPSYN306-308

                              PstI
MPSYN306 (SEQ ID NO:98) 5'GGTGGATCTAGTTCTGGAACTGTAAACCCAGCT-

                                 CCGAATATTGCCAGTCACATCTC 3',

MPSYN308 (SEQ ID NO:99) 5' GTCTATCTCCGCGAGGACTGGAGACCCAGTGAC

                                           (core) TaqI
                               GAACATGGACAT 3'

The vaccinia I3L promoter was synthesized using pMP1, a
subclone of HindIII I, as template and synthetic
oligonucleotides MPSYN310 (SEQ ID NO:100), MPSYN311 (SEQ ID
NO:101) as PCR primers. Restriction sites are indicated.
MPSYN310 (SEQ ID NO:100) 5'

              HindIII SmaI
          CCCCCCAAGCTTCCCGGGCTACATCATGCAGTGGTTAAAC 3'

                         RsaI
MPSYN311 (SEQ ID NO:101) 5' ACTTTGTAATATAATGAT 3'

    The I3 promoter/HBV S1+S2/core expression cassette was
assembled in pUC8 and pUC9 in steps, using the intermediate
plasmid clones detailed above, resulting in pMP9I3S12core.
Restriction sites are indicated only where relevant.
Plasmid pMP9I3S12core was digested with SmaI and a 1.2 kb
fragment containing the entire promoter/gene cassette was
isolated. Vaccinia HA deletion plasmid pSD544 was cut with
SmaI and ligated with the 1.2 kb fragment, producing plasmid
pMP544I3S12C.
    pMP544I3S12C was used as donor plasmid for
recombination with vaccinia recombinant vP896 described
above to generate recombinant vaccinia virus vP919. vP919
contains all three HBV inserts: spsAg, lpsAg and S12/core
fusion in the NYVAC background. The sequence of all HBV
insertions in vP919 was confirmed by polymerase chain
reaction (PCR) using vP919 as template, followed by dideoxy
sequencing of PCR generated material. In addition,
pMP544I3S12C was used in recombination with vP804 described
above to generate recombinant vaccinia virus vP858
containing only the HBV S12/core fusion. pMP544I3S12C was

also used in recombination with recombinant vaccinia virus
vP856 to generate recombinant vaccinia virus vP891. vP891
contains two HBV gene insertions, spsAg and S12/core.

Expression of HBV Proteins by vP919. To assay for the
various HBV proteins synthesized by the triple HBV
recombinant, metabolically labelled lysates from cells
infected with vP919 and appropriate vaccinia recombinants
containing single and double HBV gene insertions were
subjected to immunoprecipitation and analyzed by SDS-
polyacrylamide gel electrophoresis followed by
radioautography. Proteins in uninfected cells and cells
infected with vP866 (NYVAC), vP856 (spsAg), vP896 (spsAg +
lpsAg) or vP919 (spsAg, lpsAg, S12/core) were
immunoprecipitated using rabbit anti-S2 antiserum. Proteins
in additional uninfected cells and additional cells infected
with vP919 (spsAg, lpsAg, S12/core), vP858 (S12/core) or
vP866 (NYVAC) were immunoprecipitated using rabbit anti-core
antiserum. Anti-S2 serum precipitates a major protein of 33
kDa from vaccinia single recombinant vP856 containing the
gene for spsAg. This corresponds to the expected size for
the singly glycosylated form of HBV spsAg. A protein 36
kDa, corresponding to the expected size for the doubly
glycosylated form of spsAg is precipitated in lesser amount.
Anti-S2 serum precipitates the same proteins from vaccinia
double recombinant vP896, containing the genes for spsAg and
lpsAg. In addition, two larger proteins of 38 and 41 kDa
are precipitated, which correspond well to the expected
sizes of lpsAg (39 kDa unglycosylated and 42 kDa
glycosylated). All proteins precipitated by anti-S2 serum
from vP856 and vP896 are also precipitated from vaccinia HBV
triple recombinant vP919.

The predicted size for the HBV S12/core fusion protein
is 38 kDa. Rabbit anti-core antiserum precipitated a
protein of the predicted size as well as a variety of
smaller proteins from vP858, the vaccinia single recombinant
containing the HBV fusion gene S12/core. The most abundant
protein precipitated from vP858 by anti-core serum had a
size of 27 kDa. This corresponds in size to the translation
product which would be predicted if translation of the

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fusion protein gene began at the second (S2) ATG. The 29
kDa protein precipitated from vP858 may be the glycosylated
form of the 27 kDa protein. A smaller protein of 20 kDa,
corresponding in size to the translation product for core
protein alone, was also precipitated from vP858 in lesser
amounts. Vaccinia recombinant vP919, containing all three
HBV genes (spsAg, lpsAg and S12/core fusion), gave an
identical pattern to that observed with vP858 following
immunoprecipitation with anti-core antiserum. The 27 kDa
and 29 kDa proteins precipitated from vP858 and vP919 by
anti-core antiserum were, as expected, also precipitated
from vP919 by anti-S2 antiserum.

    <u>Antibody Response to vP919</u>. To test for serological
response to HBV proteins produced by vP919, the virus was
inoculated into rabbits, guinea pigs and mice. Rabbits and
guinea pigs were inoculated with 10^8 pfu recombinant
vaccinia virus vP919 in sets of two by intradermal,
subcutaneous or intramuscular route. Six weeks after the
primary inoculation, rabbits were boosted once by the same
route and dose. Seven weeks after the primary inoculation,
guinea pigs were boosted once by the same route and dose.
Groups of 12 mice were inoculated with 10^7 pfu recombinant
vaccinia virus vP919 by intradermal, subcutaneous or
intramuscular route. Seven weeks after the primary
inoculation, mice were boosted once by the same route. Sera
were collected at weekly intervals. Weekly bleedings from
each group of mice were pooled. All sera were analyzed for
antibody to HBV surface antigen using the AUSAB
radioimmunoassay kit (Abbott). All sera were analyzed for
antibody to HBV core antigen using the CORAB competitive
radioimmunoassay kit (Abbott). Assays were performed using
standard techniques. The results of these analyses are
presented in Tables 2 (rabbits), 3 (guinea pigs) and 4
(mice).

    Summarizing the results presented in Table 2, all six
rabbits exhibited an anti-core antibody response following a
single inoculation with vP919. In five of the six rabbits,
the anti-core antibody response was boosted by a second
inoculation of vP919. Four of six rabbits exhibited an anti

sAg response following a single inoculation of vP919. These
four rabbits, plus one additional rabbit, showed an increase
in the anti sAg response following the second inoculation.

Summarizing the results presented in Table 3, one
guinea pig exhibited an anti-core response following an
initial inoculation with vP919; following the boost at 7
weeks, a total of three guinea pigs showed an anti-core
response. One of these animals showed an anti-sAg antibody
response in week eight only.

Summarizing the results presented in Table 4, all three
groups of mice showed anti-core antibody responses at
various times after inoculation with vP919; two of the three
groups also showed anti-sAg responses.

AUSRIA Assay. Expression of particulate HBV surface
antigen from cells infected with HBV-containing vaccinia
recombinants was assayed using the commercially available
AUSRIA II-125 kit (Abbott Laboratories, North Chicago, IL).
Dishes containing 2×10^6 Vero cells were infected in
triplicate with recombinant vaccinia virus at 2 pfu/cell.
After 24 h, culture medium was removed, cells were washed
with 2 ml PBS and the wash combined with the medium and
centrifuged at 1000 rpm for 10 min. The supernatant was
designated the medium fraction. The cell fraction was
prepared by adding 2 ml PBS to the dish, scraping off the
cells and combining with the cell pellet from above. The
final volume of both medium and cell fractions were adjusted
to 4 ml with PBS. Cell fractions were sonicated for 2 min
before assay. Cell fractions and medium fractions were
assayed for the presence of HBV surface antigen at a 1:5
dilution using the AUSRIA kit. Samples below the cutoff
value of 2.1 x the negative control supplied in the kit were
considered negative. Output virus of cell and medium
fractions from all dishes were titered on Vero cells.
Results are shown in Table 5.

Construction of Vaccinia Recombinants Expressing the
HBV lpsAg under the Control of the EPV 42 kDa Promoter.
Vaccinia recombinant vP919 contains three distinct HBV genes
under the control of three different poxvirus promoters
which function at early times post infection. To compare

the relative strength of various poxvirus promoters
expressing a foreign gene at early times post infection in
the same vaccinia background, a sandwich ELISA assay was
developed, utilizing the rabies glycoprotein G gene as the
test gene. Using this test system, the vaccinia H6 promoter
and the vaccinia I3L promoter were found to be stronger
promoters than the cowpox u promoter. In vP919 the H6
promoter directs expression of the HBV spsAg, the I3L
promoter directs expression of the HBV S12/core fusion, and
the u promoter directs expression of the HBV lpsAg. The
relatively weak u promoter was purposely selected for
expression of HBV lpsAg, since it has been shown that
coexpression of lpsAg interferes with particle formation and
secretion of sAg or spsAg (Ou et al., 1987; Cheng et al.,
1986; McLachlan et al., 1987; Chisari et al., 1986).

     The AUSRIA radioimmunoassay kit was used to measure the
in vitro production of particles containing sAg or spsAg by
recombinant vaccinia virus expressing HBV genes.
Preliminary investigation showed that AUSRIA-reactive
particle formation and secretion occurred in vP856
(containing spsAg), vP896 (containing spsAg + lpsAg) and
vP919 (containing spsAg + lpsAg + S12/core). In vP896 and
vP919, the relative levels of secretion of AUSRIA-reactive
particles were lower than that observed with vP856.

     To determine whether formation and secretion of AUSRIA-
reactive particles could be observed in the presence of
higher levels of lpsAg expression, the lpsAg gene was placed
under the control of the entomopox (EPV) 42 kDa promoter.
By the comparative ELISA test described above, the EPV 42
kDa promoter in a vaccinia recombinant virus directed the
expression of a foreign gene at a level equivalent to that
observed with the vaccinia H6 promoter or the vaccinia I3L
promoter.

     Plasmid pMP550ulps contains the lpsAg gene under the
control of the cowpox u promoter in the vaccinia I4L
deletion locus (FIG. 12). The cowpox u promoter present in
plasmid pMP550ulps was replaced by the EPV 42 kDa promoter
as follows: Complementary oligonucleotides MPSYN371-374
were kinased at the internal 5' ends (MPSYN372; MPSYN373),

annealed, and cloned into pUC8 cut with EcoRI/BamHI, forming
plasmid pMP371/374. MPSYN371 (SEQ ID NO:102), MPSYN373 (SEQ
ID NO:103) MPSYN372 (SEQ ID NO:104), and MPSYN374 (SEQ ID
NO:105).

```
              EcoRI  BglII
MPSYN371 5' AATTCAGATCTCAAAATTGAAAATATATAATTACAATA

                                     TAAAATGGGGC 3'


MPSYN373 3'   GTCTAGAGTTTTAACTTTTATATATTAATGTTATAT

                                     TTTACCCCGTCTT 5'


MPSYN372 5' AGAATCTTTCCACCAGCAATCCTCTGGGATTCTTTCCCGACC

                                               BamHI
                                            ACCAGTTG 3'


MPSYN374 3' AGAAAGGTGGTCGTTAGGAGACCCTAAGAAAGGGCTGGTGGTC

                                            AACCTAG 5'
```

contain a 31 bp EPV 42 kDa promoter element, followed by HBV
S1 region (ATG underlined) to the BamHI site. Following DNA
sequence confirmation, the insert was isolated from
pMP371/374 by digestion with BamHI/BglII, and used to
replace the corresponding u promoter/HBV sequence in
pMP550ulps as follows: pMP550ulps was digested with BamHI
(partial)/BglII, and the appropriate 5 kb vector fragment
isolated and ligated with the BamHI/BglII fragment from
pMP371/374. In the resulting plasmid, pMP550E31lps, the HBV
lpsAg is under the control of the EPV 42 kDa promoter. The
entire sequence of the EPV 42 kDa promoter/lpsAg gene
cassette is given in FIG. 15.

     pMP550E31lps was used as donor plasmid with vaccinia
recombinant vP856, containing the spsAg gene, to generate
the double HBV recombinant vaccinia virus vP932. vP932 was
used as rescuing virus with donor plasmid pMP544I3S12C
containing the S12/core fusion to generate a triple HBV
recombinant vaccinia virus vP975. To generate a vaccinia
recombinant containing only the EPV 42 kDa promoter/lpsAg,
pMP550E31lps was used as donor plasmid with vP866,
generating recombinant vaccinia virus vP930.

     Secretion of HBV Surface Antigen In Vitro by
Recombinant Vaccinia Viruses. Dishes containing Vero cells

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were infected in triplicate with NYVAC (vP866) or
recombinant vaccinia virus expressing HBV spsAg and/or HBV
lpsAg. The relative amounts of HBV surface antigen
particles associated with infected cells were compared with
the amounts secreted into the medium using the AUSRIA II-125
kit (Table 5). The presence of HBV surface antigen in the
medium was not due to lysis of infected cells because more
than 99.8 % of viral infectivity remained cell associated.
Volumes of cell pellets and medium were equalized to allow
for direct comparison. In cells infected with recombinant
vaccinia virus vP856, expressing the spsAg, 42% of the
AUSRIA reactive surface antigen was secreted into the
medium. Coexpression of lpsAg under the control of the
relatively weak u promoter (vP896) did not dramatically
change the amount of cell associated AUSRIA reactive
material, but decreased the relative amount of secreted
material to 24% of the total. Coexpression of lpsAg under
the control of the relatively strong EPV 42 kDa promoter
(vP932) lowered the relative amount of secreted material to
6% of the total. As with vP896, coexpression of lpsAg with
spsAg in vP932 did not lower the amount of AUSRIA reactive
cell associated material. Interestingly, expression of
lpsAg alone under the control of the EPV 42 kDa promoter
(vP930) resulted in the production of a level of cell
associated AUSRIA reactive material significantly above
background for the assay, whereas expression of lpsAg under
the control of the u promoter (vP897) did not. This is most
likely due to the higher levels of spsAg or sAg produced in
vP930 infected cells due to initiation at internal (S2 or S)
initiation codons.

Table 2.　　Analysis of sera of rabbits inoculated with vP919[a]

CORAB[b]

#	route	week 0	1	2	3	4	5	6	7	8	9	10
A074	ID	-	20	5	5	20	5	5	80	80	20	20
A075	ID	-	20	5	5	5	5	5	80	20	20	20
A076	IM	-	5	5	1	5	5	1	80	80	80	20
A077	IM	-	1	1	1	1	1	1	80	80	80	20
A078	SC	-	1	1	-	1	1	1	20	20	5	20
A079	SC	1	5	5	1	5	5	5	5	5	5	5

AUSAB[c]

#	route	week 0	1	2	3	4	5	6	7	8	9	10
A074	ID	-	-	-	-	51	157	145	9883	3083	3483	3783
A075	ID	-	72	42	31	16	57	88	11192	4292	2792	1592
A076	IM	-	3	7	23	23	15	7	1197	200	67	63
A077	IM	-	-	-	-	-	-	-	332	127	20	20
A078	SC	-	-	-	8	-	-	-	13	-	-	-
A079	SC	1	24	32	50	80	76	540	4600	4300	4600	2800

[a]　Sets of two rabbits were inoculated with 10^8 pfu vaccinia recombinant vP919 at week 0 by intradermal (ID), intramuscular (IM) or subcutaneous (SC) route, and boosted by the same route and dose at week 6.

[b]　Undiluted sera and sera diluted 1:5, 1:20, 1:80, 1:320 with PBS were analyzed by CORAB competitive radioimmunoassay kit (Abbott). The reciprocal of the highest dilution giving a positive score is indicated. "-" indicates that undiluted serum gave a negative score.

[c]　Sera were analyzed by AUSAB radioimmunoassay kit (Abbott). Titers are expressed in RIA units/ml. "-" indicates a negative test result.

Table 3. Analysis of sera of guinea pigs inoculated with vP919[a]

CORAB[b]

#	route	week											
		0	1	2	3	4	5	6	7	8	9	10	11
047	ID	-	-	-	-	-	-	-	5	5	5	5	
048	ID	-	-	-	-	-	5	5	5[c]	5	5	5	
049	IM	-	-	-	-	-	-	-	-	-	-	-	
050	IM	-	-	-	-	-	-	-	-	-	-	-	
051	SC	-	-	-	-	-	-	-	-	5	5	5	
052	SC	-	-	-	-	-	-	-	-	-	-	-	

a   Sets of two guinea pigs were inoculated with 10^8 pfu vaccinia recombinant
    vP919 at week 0 by intradermal (ID), intramuscular (IM) or subcutaneous
    (SC) route, and boosted by the same route and dose at week 7.

b   Sera were diluted 1:5 with PBS and analyzed by CORAB competitive
    radioimmunoassay kit (Abbott) "5" indicates a positive score;
    "-" indicates a negative score.

c   1:5 dilution of this serum tested borderline positive for antibody to
    HBV surface antigen using the AUSAB radioimmunoassay kit. All other
    sera were negative by AUSAB analysis.

Table 4. Analysis of sera of mice inoculated with vP919[a]

CORAB[b] route	week										
	0	1	2	3	4	5	6	7	8	9	10
ID	-	-	-	-	-	-	5	-	5	-	5
IM	-	-	-	-	-	-	-	-	5	20	5
SC	-	-	-	-	-	5	-	5	5	-	5
AUSAB[c]											
ID	-	-	-	-	-	-	-	-	+	-	24
IM	-	-	-	-	+	10	10	+	54	13	50
SC	-	-	-	-	-	-	-	-	-	-	-

[a] Groups of 12 mice were inoculated with 10^7 pfu vaccinia recombinant vP919 at week 0 by intradermal (ID), intramuscular (IM) or subcutaneous (SC) route, and boosted by the same route and dose at week 7. Sera from each group were pooled for analysis.

[b] Sera diluted 1:5, 1:20 and 1:80 with PBS were analyzed by CORAB competitive radioimmunoassay kit (Abbott). The reciprocal of the highest dilution giving a positive score is indicated. "-" indicates that serum diluted 1:5 gave a negative score.

[c] Sera were analyzed by AUSAB radioimmunoassay kit (Abbott). Titers are expressed in RIA units/ml. "+" indicates borderline positive titer; "-" indicates a negative test result.

Table 5. HBV surface antigen expression by recombinant vaccinia virus[a]

Virus vP866	HBV genes	AUSRIA[b]				VIRUS TITER[c]		
		Cell	Medium	Total	% secreted	Cell	Medium	% released
	-	*[d]	*			1.2×10^{10}	1.7×10^7	0.15%
vP856	(H6)spsAg	96645	69305	165950	42%	1.1×10^{10}	1.5×10^7	0.14%
vP897b	(U)lpsAg	*	*			8.4×10^9	6.8×10^6	0.08%
vP896	(H6)spsAg (U)lpsAg	94751	30020	124771	24%	1.3×10^{10}	1.7×10^7	0.13%
vP930	(42 kDa)lpsag	5470	*			8.0×10^9	1.6×10^7	0.20%
vP932	(H6)spsAg (42 kDa)lpsAg	110808	6581	117389	6%	1.3×10^{10}	1.6×10^7	0.13%

a   Dishes containing 2×10^6 Vero cells were infected in triplicate at 2 pfu/cell with recombinant vaccinia virus containing HBV genes as indicated or with control virus vP866. At 24 h pi, cell and medium fractions were harvested separately, and volumes equalized to 4 ml/fraction with PBS.

b   Individual cell and medium fractions were assayed at a 1:5 dilution in PBS using the AUSRIA II-125 kit (Abbott Laboratories). Values for "Cell" and "Medium" represent the mean AUSRIA values in cpm for cell-associated and secreted HBV surface antigen, respectively, multiplied by the dilution factor. Appropriate background cpm corresponding to mean AUSRIA values for cell associated or medium fractions from vP866 infected samples were subtracted from mean AUSRIA values for the corresponding fractions from the recombinant HBV vaccinia virus infections. Values for "Total" represent the sum of the cell associated and secreted AUSRIA values multiplied by the dilution factor.

c   Output virus was titered as pfu on Vero cells. Values for "Cell" and "Medium" represent total mean cell associated and released virus per dish, respectively.

*d   AUSRIA values obtained were below the cutoff value of 2.1 x negative control as defined by the AUSRIA II-125 kit.

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Example 14 - CONSTRUCTION OF NYVAC RECOMBINANTS EXPRESSING HEPATITIS B VIRUS AND EPSTEIN BARR VIRUS

Since Epstein Barr Virus (EBV) and Hepatitis B Virus (HBV) are endemic over similar geographical areas, including Africa, it would be advantageous to produce a recombinant vaccinia virus expressing immunogens for both pathogens. To this end, vP941, a recombinant vaccinia virus containing three EBV genes and three HBV genes in a NYVAC background was generated.

Immunoprecipitation of HBV Proteins. Metabolic labelling and immunoprecipitation of HBV proteins were as described for vP919 in Example 13 with the following modifications. Infections with recombinant vaccinia virus, parental NYVAC virus (vP866) and mock infections were performed on RK-13 cells, rather than Vero cells. Both anti-S2 and anti-core antisera were preadsorbed with vP866 infected RK-13 cells.

Generation of Recombinant Vaccinia Virus vP941. Plasmid EBV Triple.1, the donor plasmid containing three EBV genes which was used to generate the vaccinia virus recombinant EBV triplet vP944, was used in recombination with vP919, the vaccinia virus recombinant HBV triplet, as rescuing virus. The resulting virus, vP941, was identified by ^{32}P-labelled EBV DNA. Like vP944, vP941 contained EBV genes gH under the control of the Entomopox virus 42 kDa promoter, gB under the control of the vaccinia H6 promoter and gp340 under the control of the vaccinia H6 promoter, all inserted in the vaccinia TK deletion locus. Like vP919, vP941 contained the synthetic HBV spsAg under the control of the vaccinia H6 promoter inserted into the ATI deletion locus, the HBV lpsAg under the control of the cowpox u promoter inserted into the I4L deletion locus, and the HBV S12/core fusion gene under the control of the I3L promoter inserted into the HA deletion locus. The integrity of the genome of recombinant vaccinia virus vP941 was confirmed by restriction analysis of the DNA.

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Expression of HBV Proteins by vP941. To assay for the various HBV proteins synthesized by sextuplet HBV/EBV vaccinia recombinant vP941, metabolically labelled proteins synthesized in RK-13 cells infected with vP941 and appropriate single, double and triple HBV recombinants were subjected to immunoprecipitation. Proteins in uninfected cells and cells infected with vP866 (NYVAC), vP856 (spsAg), vP896 (spsAg + lpsAg), vP919 (spsAg + lpsAg + S12/core), or vP941 were immunoprecipitated using rabbit anti-S2 antiserum. Proteins in additional uninfected cells and additional cells infected with vP941, vP919, vP858 (S12/core), or vP866 were immunoprecipitated using anti-core antiserum.

Anti-S2 serum precipitates two proteins of 33 kDa and 36 kDa from vaccinia single recombinant vP856 containing the gene for spsAg. These correspond to the expected sizes for the singly and doubly glycosylated forms of HBV spsAg. Anti-S2 serum precipitates the same proteins from vaccinia double recombinant vP896, containing the genes for spsAg and lpsAg. In addition, a protein of 42 kDa, corresponding to the singly glycosylated form of lpsAg is precipitated, as well as larger proteins of 45 kDa and 48 kDa. The 39 kDa protein corresponding to the nonglycosylated form of lpsAg is precipitated in minor amounts compared to the glycosylated forms. All proteins precipitated by anti-S2 serum from vP856 and vP896 are also precipitated from HBV triple recombinant vP919 and the HBV/EBV sextuplet, vP941. In the radioautogram, HBV proteins are immunoprecipitated by anti-S2 serum from RK-13 cells infected with vaccinia recombinants. When HBV proteins were immunoprecipitated from Vero cells infected with the same vaccinia recombinants (vP856, vP896 and vP919) the same proteins were observed but in different relative amounts. In general, both spsAg and lpsAg expressed by these recombinant vaccinia virus seems to be more fully glycosylated in RK-13 cells than in Vero cells.

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As was seen with Vero cells infected with vP858, the most abundant protein precipitated by anti-core serum from RK-13 cells infected with vP858 has a size of 27 kDa. This corresponds to the size of the translation product which would be predicted if translation of the S12/core fusion gene began at the second (S2) ATG. Unlike the situation observed following vP858 infection of Vero cells, vP858 infection of RK-13 cells followed by immunoprecipitation with anti-core serum does not result in a visible band corresponding in size to the 38 kDa expected for the complete S12/core translation product. All proteins precipitated by anti-core serum from HBV single recombinant vP858 are also precipitated from HBV triple recombinant vP919 and HBV/EBV sextuplet vP941.

Example 15 - CONSTRUCTION OF ALVAC RECOMBINANTS EXPRESSING RABIES VIRUS GLYCOPROTEIN G

This example describes the development of a canarypox-rabies recombinant designated as ALVAC-RG (vCP65) and its safety and efficacy.

Cells and Viruses. The parental canarypox virus (Rentschler strain) is a vaccinal strain for canaries. The vaccine strain was obtained from a wild type isolate and attenuated through more than 200 serial passages on chick embryo fibroblasts. A master viral seed was subjected to four successive plaque purifications under agar and one plaque clone was amplified through five additional passages after which the stock virus was used as the parental virus in in vitro recombination tests. The plaque purified canarypox isolate is designated ALVAC.

Construction of a Canarypox Insertion Vector. An 880 bp canarypox PvuII fragment was cloned between the PvuII sites of pUC9 to form pRW764.5. The sequence of this fragment is shown in FIG. 16 between positions 1372 and 2251. The limits of an open reading frame designated as C5 were defined. It was determined that the open reading frame was initiated at position 166 within the fragment and

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terminated at position 487. The C5 deletion was made
without interruption of open reading frames. Bases from
position 167 through position 455 were replaced with the
sequence (SEQ ID NO:106) GCTTCCCGGGAATTCTAGCTAGCTAGTTT.
This replacement sequence contains HindIII, SmaI and EcoRI
insertion sites followed by translation stops and a
transcription termination signal recognized by vaccinia
virus RNA polymerase (Yuen et al., 1987). Deletion of the
C5 ORF was performed as described below. Plasmid pRW764.5
was partially cut with RsaI and the linear product was
isolated. The RsaI linear fragment was recut with BglII and
the pRW764.5 fragment now with a RsaI to BglII deletion from
position 156 to position 462 was isolated and used as a
vector for the following synthetic oligonucleotides:
RW145 (SEQ ID NO:107):
ACTCTCAAAAGCTTCCCGGGAATTCTAGCTAGCTAGTTTTTTATAAA
RW146 (SEQ ID NO:108):
GATCTTTATAAAAACTAGCTAGCTAGAATTCCCGGGAAGCTTTTGAGAGT
Oligonucleotides RW145 and RW146 were annealed and inserted
into the pRW 764.5 RsaI and BglII vector described above.
The resulting plasmid is designated pRW831.
     Construction of Insertion Vector Containing the Rabies
G Gene. Construction of pRW838 is illustrated below.
Oligonucleotides A through E, which overlap the translation
initiation codon of the H6 promoter with the ATG of rabies
G, were cloned into pUC9 as pRW737. Oligonucleotides A
through E contain the H6 promoter, starting at NruI, through
the HindIII site of rabies G followed by BglII. Sequences
of oligonucleotides A through E (SEQ ID NO:109)-(SEQ ID NO.
113) are:
     A (SEQ ID NO:109): CTGAAATTATTTCATTATCGCGATATCCGTTAA
                GTTTGTATCGTAATGGTTCCTCAGGCTCTCCTGTTTGT
     B (SEQ ID NO:110): CATTACGATACAAACTTAACGGATATCGCGATAA
                TGAAATAATTTCAG

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C (SEQ ID NO:111): ACCCCTTCTGGTTTTTCCGTTGTGTTTT
                    GGGAAATTCCCTATTTACACGATCCCAGACA
                    AGCTTAGATCTCAG

D (SEQ ID NO:112): CTGAGATCTAAGCTTGTCTGGGATCGTGTAAATA
                    GGGAATTTCCCAAAACA

E (SEQ ID NO:113): CAACGGAAAAACCAGAAGGGGTACAAACAGGAGA
                    GCCTGAGGAAC

The diagram of annealed oligonucleotides A through E is as
follows:

```
                  A                            C
    --------------------------|--------------------------
    ----------------|--------------------|---------------
          B                    E                   D
```
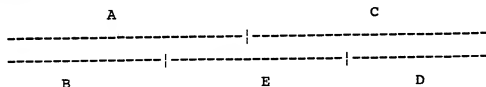
Oligonucleotides A through E were kinased, annealed
(95°C for 5 minutes, then cooled to room temperature), and
inserted between the PvuII sites of pUC9. The resulting
plasmid, pRW737, was cut with HindIII and BglII and used as
a vector for the 1.6 kbp HindIII-BglII fragment of ptg155PRO
(Kieny et al., 1984) generating pRW739. The ptg155PRO
HindIII site is 86 bp downstream of the rabies G translation
initiation codon. BglII is downstream of the rabies G
translation stop codon in ptg155PRO. pRW739 was partially
cut with NruI, completely cut with BglII, and a 1.7 kbp
NruI-BglII fragment, containing the 3' end of the H6
promoter previously described (Taylor et al., 1988a,b; Guo
et al., 1989; Perkus et al., 1989) through the entire rabies
G gene, was inserted between the NruI and BamHI sites of
pRW824. The resulting plasmid is designated pRW832.
Insertion into pRW824 added the H6 promoter 5' of NruI. The
pRW824 sequence of BamHI followed by SmaI is: GGATCCCCGGG.
pRW824 is a plasmid that contains a nonpertinent gene linked
precisely to the vaccinia virus H6 promoter. Digestion with
NruI and BamHI completely excised this nonpertinent gene.
The 1.8 kbp pRW832 SmaI fragment, containing H6 promoted
rabies G, was inserted into the SmaI of pRW831, to form
plasmid pRW838.

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Development of ALVAC-RG. Plasmid pRW838 was
transfected into ALVAC infected primary CEF cells by using
the calcium phosphate precipitation method previously
described (Panicali et al., 1982; Piccini et al., 1987).
Positive plaques were selected on the basis of hybridization
to a specific rabies G probe and subjected to 6 sequential
rounds of plaque purification until a pure population was
achieved. One representative plaque was then amplified and
the resulting ALVAC recombinant was designated ALVAC-RG
(vCP65). The correct insertion of the rabies G gene into
the ALVAC genome without subsequent mutation was confirmed
by sequence analysis.

     Immunofluorescence. During the final stages of
assembly of mature rabies virus particles, the glycoprotein
component is transported from the golgi apparatus to the
plasma membrane where it accumulates with the carboxy
terminus extending into the cytoplasm and the bulk of the
protein on the external surface of the cell membrane. In
order to confirm that the rabies glycoprotein expressed in
ALVAC-RG was correctly presented, immunofluorescence was
performed on primary CEF cells infected with ALVAC or ALVAC-
RG. Immunofluorescence was performed as previously
described (Taylor et al., 1990) using a rabies G monoclonal
antibody. Strong surface fluorescence was detected on CEF
cells infected with ALVAC-RG but not with the parental
ALVAC.

     Immunoprecipitation. Preformed monolayers of primary
CEF, Vero (a line of African Green monkey kidney cells ATCC
CCL81) and MRC-5 cells (a fibroblast-like cell line
derived from normal human fetal lung tissue ATCC # CCL171)
were inoculated at 10 pfu per cell with parental virus ALVAC
and recombinant virus ALVAC-RG in the presence of
radiolabelled ^{35}S-methionine and treated as previously
described (Taylor et al., 1990). Immunoprecipitation
reactions were performed using a rabies G specific
monoclonal antibody. Efficient expression of a rabies

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specific glycoprotein with a molecular weight of
approximately 67 kDa was detected with the recombinant
ALVAC-RG. No rabies specific products were detected in
uninfected cells or cells infected with the parental ALVAC
virus.

Sequential Passaging Experiment. In studies with ALVAC
virus in a range of non-avian species no proliferative
infection or overt disease was observed (Taylor et al.,
1991b). However, in order to establish that neither the
parental nor recombinant virus could be adapted to grow in
non-avian cells, a sequential passaging experiment was
performed.

The two viruses, ALVAC and ALVAC-RG, were inoculated in
10 sequential blind passages in three cell lines:

(1)   Primary chick embryo fibroblast (CEF) cells
      produced from 11 day old white leghorn embryos;

(2)   Vero cells - a continuous line of African Green
      monkey kidney cells (ATCC # CCL81); and

(3)   MRC-5 cells - a diploid cell line derived from
      human fetal lung tissue (ATCC # CCL171).

The initial inoculation was performed at an m.o.i. of 0.1
pfu per cell using three 60mm dishes of each cell line
containing 2 X 10^6 cells per dish. One dish was inoculated
in the presence of 40μg/ml of Cytosine arabinoside (Ara C),
an inhibitor of DNA replication. After an absorption period
of 1 hour at 37°C, the inoculum was removed and the
monolayer washed to remove unabsorbed virus. At this time
the medium was replaced with 5ml of EMEM + 2% NBCS on two
dishes (samples t0 and t7) and 5ml of EMEM + 2% NBCS
containing 40 μg/ml Ara C on the third (sample t7A). Sample
t0 was frozen at -70°C to provide an indication of the
residual input virus. Samples t7 and t7A were incubated at
37°C for 7 days, after which time the contents were
harvested and the cells disrupted by indirect sonication.

One ml of sample t7 of each cell line was inoculated
undiluted onto three dishes of the same cell line (to

provide samples t0, t7 and t7A) and onto one dish of primary
CEF cells. Samples t0, t7 and t7A were treated as for
passage one. The additional inoculation on CEF cells was
included to provide an amplification step for more sensitive
detection of virus which might be present in the non-avian
cells.

This procedure was repeated for 10 (CEF and MRC-5) or 8
(Vero) sequential blind passages. Samples were then frozen
and thawed three times and assayed by titration on primary
CEF monolayers.

Virus yield in each sample was then determined by
plaque titration on CEF monolayers under agarose.
Summarized results of the experiment are shown in Tables 6
and 7.

The results indicate that both the parental ALVAC and
the recombinant ALVAC-RG are capable of sustained
replication on CEF monolayers with no loss of titer. In
Vero cells, levels of virus fell below the level of
detection after 2 passages for ALVAC and 1 passage for
ALVAC-RG. In MRC-5 cells, a similar result was evident, and
no virus was detected after 1 passage. Although the results
for only four passages are shown in Tables 6 and 7 the
series was continued for 8 (Vero) and 10 (MRC-5) passages
with no detectable adaptation of either virus to growth in
the non-avian cells.

In passage 1 relatively high levels of virus were
present in the t7 sample in MRC-5 and Vero cells. However
this level of virus was equivalent to that seen in the t0
sample and the t7A sample incubated in the presence of
Cytosine arabinoside in which no viral replication can
occur. This demonstrated that the levels of virus seen at 7
days in non-avian cells represented residual virus and not
newly replicated virus.

In order to make the assay more sensitive, a portion of
the 7 day harvest from each cell line was inoculated onto a
permissive CEF monolayer and harvested at cytopathic effect

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(CPE) or at 7 days if no CPE was evident. The results of
this experiment are shown in Table 8. Even after
amplification through a permissive cell line, virus was only
detected in MRC-5 and Vero cells for two additional
passages. These results indicated that under the conditions
used, there was no adaptation of either virus to growth in
Vero or MRC-5 cells.

     Inoculation of Macaques. Four HIV seropositive
macaques were initially inoculated with ALVAC-RG as
described in Table 9. After 100 days these animals were re-
inoculated to determine a booster effect, and an additional
seven animals were inoculated with a range of doses. Blood
was drawn at appropriate intervals and sera analyzed, after
heat inactivation at 56°C for 30 minutes, for the presence
of anti-rabies antibody using the Rapid Fluorescent Focus
Inhibition Assay (Smith et al., 1973).

     Inoculation of Chimpanzees. Two adult male chimpanzees
(50 to 65 kg weight range) were inoculated intramuscularly
or subcutaneously with 1 X 10^7 pfu of vCP65. Animals were
monitored for reactions and bled at regular intervals for
analysis for the presence of anti-rabies antibody with the
RFFI test (Smith et al., 1973). Animals were re-inoculated
with an equivalent dose 13 weeks after the initial
inoculation.

     Inoculation of Mice. Groups of mice were inoculated
with 50 to 100 μl of a range of dilutions of different
batches of vCP65. Mice were inoculated in the footpad. On
day 14, mice were challenged by intracranial inoculation of
from 15 to 43 mouse LD_{50} of the virulent CVS strain of
rabies virus. Survival of mice was monitored and a
protective dose 50% (PD_{50}) calculated at 28 days post-
inoculation.

     Inoculation of Dogs and Cats. Ten beagle dogs, 5
months old, and 10 cats, 4 months old, were inoculated
subcutaneously with either 6.7 or 7.7 $\log_{10}$ $TCID_{50}$ of ALVAC-
RG. Four dogs and four cats were not inoculated. Animals

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were bled at 14 and 28 days post-inoculation and anti-rabies
antibody assessed in an RFFI test. The animals receiving
6.7 log$_{10}$ TCID$_{50}$ of ALVAC-RG were challenged at 29 days
post-vaccination with 3.7 log$_{10}$ mouse LD$_{50}$ (dogs) or 4.3
log$_{10}$ mouse LD$_{50}$ (cats) of the NYGS rabies virus challenge
strain.

    Inoculation of Squirrel Monkeys. Three groups of four
squirrel monkeys (Saimiri sciureus) were inoculated with one
of three viruses (a) ALVAC, the parental canarypox virus,
(b) ALVAC-RG, the recombinant expressing the rabies G
glycoprotein or (c) vCP37, a canarypox recombinant
expressing the envelope glycoprotein of feline leukemia
virus. Inoculations were performed under ketamine
anaesthesia. Each animal received at the same time: (1) 20
μl instilled on the surface of the right eye without
scarification; (2) 100 μl as several droplets in the mouth;
(3) 100 μl in each of two intradermal injection sites in the
shaven skin of the external face of the right arm; and (4)
100 μl in the anterior muscle of the right thigh.

    Four monkeys were inoculated with each virus, two with
a total of 5.0 log$_{10}$ pfu and two with a total of 7.0 log$_{10}$
pfu. Animals were bled at regular intervals and sera
analyzed for the presence of antirabies antibody using an
RFFI test (Smith et al., 1973). Animals were monitored
daily for reactions to vaccination. Six months after the
initial inoculation the four monkeys receiving ALVAC-RG, two
monkeys initially receiving vCP37, and two monkeys initially
receiving ALVAC, as well as one naive monkey were inoculated
with 6.5 log$_{10}$ pfu of ALVAC-RG subcutaneously. Sera were
monitored for the presence of rabies neutralizing antibody
in an RFFI test (Smith et al., 1973).

    Inoculation of Human Cell Lines with ALVAC-RG. In
order to determine whether efficient expression of a foreign
gene could be obtained in non-avian cells in which the virus
does not productively replicate, five cell types, one avian
and four non-avian, were analyzed for virus yield,

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expression of the foreign rabies G gene and viral specific
DNA accumulation. The cells inoculated were:

    (a)   Vero, African Green monkey kidney cells, ATCC #
        CCL81;

    (b)   MRC-5, human embryonic lung, ATCC # CCL 171;

    (c)   WISH human amnion, ATCC # CCL 25;

    (d)   Detroit-532, human foreskin, Downs's syndrome,
        ATCC # CCL 54; and

    (e)   Primary CEF cells.

    Chicken embryo fibroblast cells produced from 11 day
old white leghorn embryos were included as a positive
control. All inoculations were performed on preformed
monolayers of 2×10^6 cells as discussed below.

A.   Methods for DNA analysis.

    Three dishes of each cell line were inoculated at 5
pfu/cell of the virus under test, allowing one extra
dish of each cell line un-inoculated. One dish was
incubated in the presence of 40 μg/ml of cytosine
arabinoside (Ara C). After an adsorption period of 60
minutes at 37°C, the inoculum was removed and the
monolayer washed twice to remove unadsorbed virus.
Medium (with or without Ara C) was then replaced.
Cells from one dish (without Ara C) were harvested as a
time zero sample. The remaining dishes were incubated
at 37°C for 72 hours, at which time the cells were
harvested and used to analyze DNA accumulation. Each
sample of 2×10^6 cells was resuspended in 0.5 ml
phosphate buffered saline (PBS) containing 40 mM EDTA
and incubated for 5 minutes at 37°C. An equal volume
of 1.5% agarose prewarmed at 42°C and containing 120 mM
EDTA was added to the cell suspension and gently mixed.
The suspension was transferred to an agarose plug mold
and allowed to harden for at least 15 min. The agarose
plugs were then removed and incubated for 12-16 hours
at 50°C in a volume of lysis buffer (1% sarkosyl, 100
μg/ml proteinase K, 10 mM Tris HCl pH 7.5, 200 mM EDTA)

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that completely covers the plug. The lysis buffer was
then replaced with 5.0 ml sterile 0.5 X TBE (44.5 mM
Tris-borate, 44.5 mM boric acid, 0.5 mM EDTA) and
equilibrated at 4°C for 6 hours with 3 changes of TBE
buffer. The viral DNA within the plug was fractionated
from cellular RNA and DNA using a pulse field
electrophoresis system. Electrophoresis was performed
for 20 hours at 180 V with a ramp of 50-90 sec at 15°C
in 0.5 X TBE. The DNA was run with lambda DNA
molecular weight standards. After electrophoresis the
viral DNA band was visualized by staining with ethidium
bromide. The DNA was then transferred to a
nitrocellulose membrane and probed with a radiolabelled
probe prepared from purified ALVAC genomic DNA.

B.   Estimation of virus yield.
      Dishes were inoculated exactly as described above, with
      the exception that input multiplicity was 0.1 pfu/cell.
      At 72 hours post infection, cells were lysed by three
      successive cycles of freezing and thawing. Virus yield
      was assessed by plaque titration on CEF monolayers.

C.   Analysis of expression of Rabies G gene.
      Dishes were inoculated with recombinant or parental
      virus at a multiplicity of 10 pfu/cell, allowing an
      additional dish as an uninfected virus control. After
      a one hour absorption period, the medium was removed
      and replaced with methionine free medium. After a 30
      minute period, this medium was replaced with
      methionine-free medium containing 25 uCi/ml of ^{35}S-
      Methionine. Infected cells were labelled overnight
      (approximately 16 hours), then lysed by the addition of
      buffer A lysis buffer. Immunoprecipitation was
      performed as previously described (Taylor et al., 1990)
      using a rabies G specific monoclonal antibody.
      Results: Estimation of Viral Yield. The results of
titration for yield at 72 hours after inoculation at 0.1 pfu
per cell are shown in Table 10. The results indicate that

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while a productive infection can be attained in the avian
cells, no increase in virus yield can be detected by this
method in the four non-avian cell systems.

Analysis of Viral DNA Accumulation. In order to
determine whether the block to productive viral replication
in the non-avian cells occurred before or after DNA
replication, DNA from the cell lysates was fractionated by
electrophoresis, transferred to nitrocellulose and probed
for the presence of viral specific DNA. DNA from uninfected
CEF cells, ALVAC-RG infected CEF cells at time zero, ALVAC-
RG infected CEF cells at 72 hours post-infection and ALVAC-
RG infected CEF cells at 72 hours post-infection in the
presence of 40 μg/ml of cytosine arabinoside all showed some
background activity, probably due to contaminating CEF
cellular DNA in the radiolabelled ALVAC DNA probe
preparation. However, ALVAC-RG infected CEF cells at 72
hours post-infection exhibited a strong band in the region
of approximately 350 kbp representing ALVAC-specific viral
DNA accumulation. No such band is detectable when the
culture is incubated in the presence of the DNA synthesis
inhibitor, cytosine arabinoside. Equivalent samples
produced in Vero cells showed a very faint band at
approximately 350 kbp in the ALVAC-RG infected Vero cells at
time zero. This level represented residual virus. The
intensity of the band was amplified at 72 hours post-
infection indicating that some level of viral specific DNA
replication had occurred in Vero cells which had not
resulted in an increase in viral progeny. Equivalent
samples produced in MRC-5 cells indicated that no viral
specific DNA accumulation was detected under these
conditions in this cell line. This experiment was then
extended to include additional human cell lines,
specifically WISH and Detroit-532 cells. ALVAC infected CEF
cells served as a positive control. No viral specific DNA
accumulation was detected in either WISH or Detroit cells
inoculated with ALVAC-RG. It should be noted that the

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limits of detection of this method have not been fully
ascertained and viral DNA accumulation may be occurring, but
at a level below the sensitivity of the method. Other
experiments in which viral DNA replication was measured by
^{3}H-thymidine incorporation support the results obtained with
Vero and MRC-5 cells.

    Analysis of Rabies Gene Epression. To determine if any
viral gene expression, particularly that of the inserted
foreign gene, was occurring in the human cell lines even in
the absence of viral DNA replication, immunoprecipitation
experiments were performed on ^{35}S-methionine labelled
lysates of avian and non-avian cells infected with ALVAC and
ALVAC-RG. The results of immunoprecipitation using a rabies
G specific monoclonal antibody illustrated specific
immunoprecipitation of a 67 kDa glycoprotein in CEF, Vero
and MRC-5, WISH and Detroit cells infected with ALVAC-RG.
No such specific rabies gene products were detected in any
of the uninfected and parentally infected cell lysates.

    The results of this experiment indicated that in the
human cell lines analyzed, although the ALVAC-RG recombinant
was able to initiate an infection and express a foreign gene
product under the transcriptional control of the H6
early/late vaccinia virus promoter, the replication did not
proceed through DNA replication, nor was there any
detectable viral progeny produced. In the Vero cells,
although some level of ALVAC-RG specific DNA accumulation
was observed, no viral progeny was detected by these
methods. These results would indicate that in the human
cell lines analyzed the block to viral replication occurs
prior to the onset of DNA replication, while in Vero cells,
the block occurs following the onset of viral DNA
replication.

    In order to determine whether the rabies glycoprotein
expressed in ALVAC-RG was immunogenic, a number of animal
species were tested by inoculation of the recombinant. The
efficacy of current rabies vaccines is evaluated in a mouse

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model system. A similar test was therefore performed using
ALVAC-RG. Nine different preparations of virus (including
one vaccine batch (J) produced after 10 serial tissue
culture passages of the seed virus) with infectious titers
ranging from 6.7 to 8.4 $\log_{10}$ $TCID_{50}$ per ml were serially
diluted and 50 to 100 μl of dilutions inoculated into the
footpad of four to six week old mice. Mice were challenged
14 days later by the intracranial route with 300 μl of the
CVS strain of rabies virus containing from 15 to 43 mouse
LD_{50} as determined by lethality titration in a control group
of mice. Potency, expressed as the PD_{50} (Protective dose
50%), was calculated at 14 days post-challenge. The results
of the experiment are shown in Table 11. The results
indicated that ALVAC-RG was consistently able to protect
mice against rabies virus challenge with a PD_{50} value
ranging from 3.33 to 4.56 with a mean value of 3.73 (STD
0.48). As an extension of this study, male mice were
inoculated intracranially with 50 μl of virus containing 6.0
$\log_{10}$ $TCID_{50}$ of ALVAC-RG or with an equivalent volume of an
uninfected cell suspension. Mice were sacrificed on days 1,
3 and 6 post-inoculation and their brains removed, fixed and
sectioned. Histopathological examination showed no evidence
for neurovirulence of ALVAC-RG in mice.

     In order to evaluate the safety and efficacy of ALVAC-
RG for dogs and cats, a group of 14, 5 month old beagles and
14, 4 month old cats were analyzed. Four animals in each
species were not vaccinated. Five animals received 6.7
$\log_{10}$ $TCID_{50}$ subcutaneously and five animals received 7.7
$\log_{10}$ $TCID_{50}$ by the same route. Animals were bled for
analysis for anti-rabies antibody. Animals receiving no
inoculation or 6.7 $\log_{10}$ $TCID_{50}$ of ALVAC-RG were challenged
at 29 days post-vaccination with 3.7 $\log_{10}$ mouse LD_{50} (dogs,
in the temporal muscle) or 4.3 $\log_{10}$ mouse LD_{50} (cats, in
the neck) of the NYGS rabies virus challenge strain. The
results of the experiment are shown in Table 12.

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No adverse reactions to inoculation were seen in either
cats or dogs with either dose of inoculum virus. Four of 5
dogs immunized with 6.7 $\log_{10}$ $TCID_{50}$ had antibody titers on
day 14 post-vaccination and all dogs had titers at 29 days.
All dogs were protected from a challenge which killed three
out of four controls. In cats, three of five cats receiving
6.7 $\log_{10}$ $TCID_{50}$ had specific antibody titers on day 14 and
all cats were positive on day 29 although the mean antibody
titer was low at 2.9 IU. Three of five cats survived a
challenge which killed all controls. All cats immunized
with 7.7 $\log_{10}$ $TCID_{50}$ had antibody titers on day 14 and at
day 29 the Geometric Mean Titer was calculated as 8.1
International Units.

The immune response of squirrel monkeys (*Saimiri
sciureus*) to inoculation with ALVAC, ALVAC-RG and an
unrelated canarypox virus recombinant was examined. Groups
of monkeys were inoculated as described above and sera
analyzed for the presence of rabies specific antibody.
Apart from minor typical skin reactions to inoculation by
the intradermal route, no adverse reactivity was seen in any
of the monkeys. Small amounts of residual virus were
isolated from skin lesions after intradermal inoculation on
days two and four post-inoculation only. All specimens were
negative on day seven and later. There was no local
reaction to intra-muscular injection. All four monkeys
inoculated with ALVAC-RG developed anti-rabies serum
neutralizing antibodies as measured in an RFFI test.
Approximately six months after the initial inoculation all
monkeys and one additional naive monkey were re-inoculated
by the subcutaneous route on the external face of the left
thigh with 6.5 $\log_{10}$ $TCID_{50}$ of ALVAC-RG. Sera were analyzed
for the presence of anti-rabies antibody. The results are
shown in Table 13.

Four of the five monkeys naive to rabies developed a
serological response by seven days post-inoculation with
ALVAC-RG. All five monkeys had detectable antibody by 11

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days post-inoculation. Of the four monkeys with previous
exposure to the rabies glycoprotein, all showed a
significant increase in serum neutralization titer between
days 3 and 7 post-vaccination. The results indicate that
vaccination of squirrel monkeys with ALVAC-RG does not
produce adverse side-effects and a primary neutralizing
antibody response can be induced. An amnanestic response is
also induced on re-vaccination. Prior exposure to ALVAC or
to a canarypox recombinant expressing an unrelated foreign
gene does not interfere with induction of an anti-rabies
immune response upon re-vaccination.

The immunological response of HIV-2 seropositive
macaques to inoculation with ALVAC-RG was assessed. Animals
were inoculated as described above and the presence of anti-
rabies serum neutralizing antibody assessed in an RFFI test.
The results, shown in Table 14, indicated that HIV-2
positive animals inoculated by the subcutaneous route
developed anti-rabies antibody by 11 days after one
inoculation. An anamnestic response was detected after a
booster inoculation given approximately three months after
the first inoculation. No response was detected in animals
receiving the recombinant by the oral route. In addition, a
series of six animals were inoculated with decreasing doses
of ALVAC-RG given by either the intra-muscular or
subcutaneous routes. Five of the six animals inoculated
responded by 14 days post-vaccination with no significant
difference in antibody titer.

Two chimpanzees with prior exposure to HIV were
inoculated with 7.0 $\log_{10}$ pfu of ALVAC-RG by the
subcutaneous or intra-muscular route. At 3 months post-
inoculations both animals were re-vaccinated in an identical
fashion. The results are shown in Table 15.

No adverse reactivity to inoculation was noted by
either intramuscular or subcutaneous routes. Both
chimpanzees responded to primary inoculation by 14 days and

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a strongly rising response was detected following re-
vaccination.

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Table 6. Sequential Passage of ALVAC in Avian and non-Avian
          Cells.

	CEF	Vero	MRC-5
Pass 1			
Sample to[a]	2.4	3.0	2.6
t7[b]	7.0	1.4	0.4
t7A[c]	1.2	1.2	0.4
Pass 2			
Sample to	5.0	0.4	N.D.[d]
t7	7.3	0.4	N.D.
t7A	3.9	N.D.	N.D.
Pass 3			
Sample to	5.4	0.4	N.D.
t7	7.4	N.D.	N.D.
t7A	3.8	N.D.	N.D.
Pass 4			
Sample to	5.2	N.D.	N.D.
t7	7.1	N.D.	N.D.
t7A	3.9	N.D.	N.D.

----

a: This sample was harvested at zero time and represents
     the residual input virus. The titer is expressed as
     $\log_{10}$pfu per ml.

b: This sample was harvested at 7 days post-infection.

c: This sample was inoculated in the presence of 40 µg/ml
     of Cytosine arabinoside and harvested at 7 days post
     infection.

d: Not detectable

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Table 7.   Sequential Passage of ALVAC-RG in Avian and non-
           Avian Cells

	CEF	Vero	MRC-5
Pass 1			
Sample t0[a]	3.0	2.9	2.9
t7[b]	7.1	1.0	1.4
t7A[c]	1.8	1.4	1.2
Pass 2			
Sample t0	5.1	0.4	0.4
t7	7.1	N.D.[d]	N.D.
t7A	3.8	N.D.	N.D.
Pass 3			
Sample t0	5.1	0.4	N.D.
t7	7.2	N.D.	N.D.
t7A	3.6	N.D.	N.D.
Pass 4			
Sample t0	5.1	N.D.	N.D.
t7	7.0	N.D.	N.D.
t7A	4.0	N.D.	N.D

a: This sample was harvested at zero time and represents
     the residual input virus. The titer is expressed as
     $\log_{10}$pfu per ml.

b: This sample was harvested at 7 days post-infection.

c: This sample was inoculated in the presence of 40 μg/ml
     of Cytosine arabinoside and harvested at 7 days post-
     infection.

d: Not detectable.

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Table 8. Amplification of residual virus by passage in CEF
          cells

CEF	Vero	MRC-5		
a) ALVAC				
Pass 2[a]	7.0[b]	6.0	5.2
3	7.5	4.1	4.9
4	7.5	N.D.[c]	N.D.
5	7.1	N.D.	N.D.
b) ALVAC-RG				
Pass 2[a]	7.2	5.5	5.5
3	7.2	5.0	5.1
4	7.2	N.D.	N.D.
5	7.2	N.D.	N.D.

a: Pass 2 represents the amplification in CEF cells of the
     7 day sample from Pass 1.

b: Titer expressed as $\log_{10}$ pfu per ml

c: Not Detectable

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Table 9. Schedule of inoculation of rhesus macaques with
          ALVAC-RG (vCP65)

Animal		Inoculation
176L	Primary:	1 X 10^8 pfu of vCP65 orally in TANG
	Secondary:	1 X 10^7 pfu of vCP65 plus 1 X 10^7
		pfu of vCP82[a] by SC route
185 L	Primary:	1 X 10^8 pfu of vCP65 orally in Tang
	Secondary:	1 X 10^7 pfu of vCP65 plus 1 X 10^7
		pfu of vCP82 by SC route
177 L	Primary:	5 X 10^7 pfu SC of vCP65 by SC route
	Secondary:	1 X 10^7 pfu of vCP65 plus 1 X 10^7
		pfu of vCP82 by SC route
186L	Primary:	5 X 10^7 pfu of vCP65 by SC route
	Secondary:	1 X 10^7 pfu of vCP65 plus 1 X 10^7
		pfu of vCP82 by SC route
178L	Primary:	1 X 10^7 pfu of vCP65 by SC route
182L	Primary:	1 X 10^7 pfu of vCP65 by IM route
179L	Primary:	1 X 10^6 pfu of vCP65 by SC route
183L	Primary:	1 X 10^6 pfu of vCP65 by IM route
180L	Primary:	1 X 10^6 pfu of vCP65 by SC route
184L	Primary:	1 X 10^5 pfu of vCP65 by IM route
187L	Primary	1 X 10^7 pfu of vCP65 orally

a: vCP82 is a canarypox virus recombinant expressing the
    measles virus fusion and hemagglutinin genes.

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Table 10. Analysis of yield in avian and non-avian cells

inoculated with ALVAC-RG

Sample Time Cell Type	t0	t72	t72A[b]
Expt 1			
CEF	3.3[a]	7.4	1.7
Vero	3.0	1.4	1.7
MRC-5	3.4	2.0	1.7
Expt 2			
CEF	2.9	7.5	<1.7
WISH	3.3	2.2	2.0
Detroit-532	2.8	1.7	<1.7

a: Titer expressed as $\log_{10}$ pfu per ml

b: Culture incubated in the presence of 40 μg/ml of

Cytosine arabinoside

Table 11. Potency of ALVAC-RG as tested in mice

Test	Challenge Dose[a]	PD_{50}[b]
Initial seed	43	4.56
Primary seed	23	3.34
Vaccine Batch H	23	4.52
Vaccine Batch I	23	3.33
Vaccine Batch K	15	3.64
Vaccine Batch L	15	4.03
Vaccine Batch M	15	3.32
Vaccine Batch N	15	3.39
Vaccine Batch J	23	3.42

a: Expressed as mouse LD_{50}

b: Expressed as $\log_{10} TCID_{50}$

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Table 12. Efficacy of ALVAC-RG in dogs and cats

| | Dogs | | Cats | |
Dose	Antibody[a]	Survival[b]	Antibody	Survival
6.7	11.9	5/5	2.9	3/5
7.7	10.1	N.T.	8.1	N.T.

a: Antibody at day 29 post inoculation expressed as the

     geometric mean titer in International Units.

b: Expressed as a ratio of survivors over animals

     challenged

Table 13. Anti-rabies serological response of Squirrel monkeys inoculated with canarypox recombinants

Monkey #	Previous Exposure	Rabies serum-neutralizing antibody[a]						
		-196[b]	0	3	7	11	21	28
22	ALVAC[c]	NT[g]	<1.2	<1.2	<1.2	2.1	2.3	2.2
51	ALVAC[c]	NT	<1.2	<1.2	1.7	2.2	2.2	2.2
39	vCP37[d]	NT	<1.2	<1.2	1.7	2.1	2.2	N.T.[g]
55	vCP37[d]	NT	<1.2	<1.2	1.7	2.2	2.1	N.T.
37	ALVAC-RG[e]	2.2	<1.2	<1.2	3.2	3.5	3.5	3.2
53	ALVAC-RG[e]	2.2	<1.2	<1.2	3.6	3.6	3.6	3.4
38	ALVAC-RG[f]	2.7	<1.7	<1.7	3.2	3.8	3.6	N.T.
54	ALVAC-RG[f]	3.2	<1.7	<1.5	3.6	4.2	4.0	3.6
57	None	NT	<1.2	<1.2	1.7	2.7	2.7	2.3

a: As determined by RFFI test on days indicated and expressed in International Units

b: Day-196 represents serum from day 28 after primary vaccination

c: Animals received 5.0 log10 TCID50 of ALVAC

d: Animals received 5.0 log10 TCID50 of vCP37

e: Animals received 5.0 log10 TCID50 of ALVAC-RG

f: Animals received 7.0 log10 TCID50 of ALVAC-RG

g: Not tested.

Table 14. Inoculation of rhesus macaques with ALVAC-RG[a]

Days post-Inoculation	Route of Primary Inoculation										
	or/Tang 176L[b]	185L	SC 177L	SC 186L	SC 178L	IM 182L	SC 179L	IM 183L	SC 180L	IM 184L	OR 187L[b]
-84	–	–	–	–	–	–	–	–	–	–	–
-9	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–
6	–	–	±	±	–	–	–	–	–	–	–
11	–	–	16[d]	128	–	–	–	–	–	–	–
19	–	–	32	128	–	–	–	–	–	–	–
35	–	–	32	512	–	–	–	–	–	–	–
59	–	–	64	256	–	–	–	–	–	–	–
75	–	–	64	128	–	–	–	–	–	–	–
99[c]	–	–	64	256	–	–	–	–	–	–	–
2	–	–	32	256	–	–	–	–	–	–	–
6	–	–	512	512	–	–	–	–	–	–	–
15	16	16	512	512	64	32	64	–	32	–	–
29	16	32	256	256	64	64	32	128	128	32	–
55	16	32	256	256	32	–	16	128	32	16	–
57	16	32	128	128	16	32	16	32	16	–	–

a: See Table 9 for schedule of inoculations.
b: Animals 176L and 185L received 8.0 log10 pfu by the oral route in 5 ml Tang. Animal 187L received 7.0 log10 pfu by oral route not in Tang.
c: Day of re-vaccination for animals 176L, 185L, 177L, 186L, 178L, 180L, 184L, and 187L.
d: Titers expressed as reciprocal of last dilution showing inhibition of fluorescence in an RFFI test.

Table 15.   Inoculation of chimpanzees with ALVAC-RG

Weeks post- Inoculation	Animal 431 I.M.	Animal 457 S.C.
0	<8[a]	<8
1	<8	<8
2	8	32
4	16	32
8	16	32
12[b]/0	16	8
13/1	128	128
15/3	256	512
20/8	64	128
26/12	32	128

a: Titer expressed as reciprocal of last dilution showing

     inhibition of fluorescence in an RFFI test

b: Day of re-inoculation

Example 16   -   CONSTRUCTION OF NYVAC RECOMBINANTS EXPRESSING
                FLAVIVIRUS PROTEINS

     This example describes the construction of NYVAC donor
plasmids containing genes from Japanese encephalitis virus
(JEV), yellow fever virus (YF) and Dengue type 1, the
isolation of the corresponding NYVAC Flavivirus recombinants
and the ability of vaccinia recombinants expressing portions
of the genomes of JEV or YF to protect mice against lethal
challenge with the homologous virus.

     Cell Lines and Virus Strains. A thymidine kinase
mutant of the Copenhagen strain of vaccinia virus vP410 (Guo
et al., 1989) was used to generate recombinants vP825,
vP829, vP857 and vP864 (see below). The generation of vP555
has previously been described (Mason et al., 1991).
Biosynthetic studies were performed using HeLa cells grown
at 37°C in Eagle's minimal essential medium supplemented
with FBS and antibiotics. The JEV virus used in all *in
vitro* experiments was a clarified culture fluid prepared
from C6/36 cells infected with a passage 55 suckling mouse
brain suspension of the Nakayama strain of JEV (Mason,
1989). Animal challenge experiments were performed using
the highly pathogenic P3 strain of JEV (see below).

     Cloning of JEV Genes Into a Vaccinia Virus Donor
Plasmid. The JEV cDNAs used to construct the JEV-vaccinia
recombinant viruses were derived from the Nakayama strain of
JEV (McAda et al., 1987).

     Plasmid pDr20 containing JEV cDNA (nucleotides -28 to
1000) in the SmaI and EcoRI sites of pUC18, was digested
with BamHI and EcoRI and the JEV cDNA insert cloned into
pIBI25 (International Biotechnologies, Inc., New Haven, CT)
generating plasmid JEV18. JEV18 was digested with ApaI
within the JE sequence (nucleotide 23) and XhoI within
pIBI25 and ligated to annealed oligonucleotides J90 (SEQ ID
NO:114) and J91 (SEQ ID NO:115) (containing an XhoI sticky
end, SmaI site, and JE nucleotides 1 to 23) generating
plasmid JEV19. JEV19 was digested with XhoI within pIBI25
and AccI within JE sequences (nucleotide 602) and the
resulting 613 bp fragment was cloned into the XhoI and AccI
fragment of JEV2 (Mason et al., 1991) containing the plasmid

origin and JEV cDNA encoding the carboxy-terminal 40% prM
and amino-terminal two thirds of E (nucleotides 602 to
2124), generating plasmid JEV20 containing JE sequences from
the ATG of C through the SacI site (nucleotide 2124) found
in the last third of E.

The SmaI-SacI fragment from JEV8 (a plasmid analogous
to JEVL Mason et al., 1991) in which TTTTTGT nucleotides
1304 to 1310 were changed to TCTTTGT), containing JE
sequences from the last third of E through the first two
amino acids of NS2B (nucleotides 2124 to 4126), the plasmid
origin and vaccinia sequences, was ligated to the purified
SmaI-SacI insert from JEV20 yielding JEV22-1. The 6 bp
corresponding to the unique SmaI site used to construct
JEV22-1 were removed using oligonucleotide-directed double-
strand break mutagenesis (Mandecki, 1986) creating JEV24 in
which the H6 promoter immediately preceded the ATG start
codon.

Plasmid JEV7 (Mason et al., 1991) was digested with
SphI within JE sequences (nucleotide 2180) and HindIII
within IBI24. Ligation to annealed oligonucleotides J94 and
J95 [containing SphI sticky end, translation stop, and a
vaccinia early transcription termination signal (TTTTTAT;
Yuen et al., 1987) a translation stop, an EagI site and a
HindIII sticky end] generated plasmid JEV25 which contains
JE cDNA extending from the SacI site (nucleotide 2124) in
the last third of E through the carboxy-terminus of E. The
SacI-EagI fragment from JEV25 was ligated to the SacI-EagI
fragment of JEV8 (containing JE cDNA encoding 15 aa C, prM
and amino-terminal two thirds of E nucleotides 337 to 2124,
the plasmid origin and vaccinia sequences) yielding plasmid
JEV26. A unique SmaI site preceding the ATG start codon was
removed as described above, creating JEV27 in which the H6
promoter immediately preceded the ATG start codon.

Oligonucleotides J96, J97, J98 and J99 (containing JE
nucleotides 2243 to 2380 with an SphI sticky end) were
kinased, annealed and ligated to SmaI-SphI digested and
alkaline phosphatase treated pIBI25 generating plasmid
JEV28. JEV28 was digested with HpaI within the JE sequence
(nucleotide 2301) and with HindIII within the pIBI25

sequence and alkaline phosphatase treated. Ligation to the
HpaI-HindIII fragment from JEV1 or HpaI-HindIII fragment
from JEV7 (Mason et al., 1991) yielded JEV29 (containing a
SmaI site followed by JE cDNA encoding 30 aa E, NS1, NS2A
nucleotides 2293 to 4126) and JEV30 (containing a SmaI site
followed by JE cDNA encoding 30 aa E, NS1, NS2A, NS2B
nucleotides 2293 to 4512).

     The SmaI-EagI fragment from JEV29 was ligated to SmaI-
EagI digested pTP15 (Mason et al., 1991) yielding JEV31.
The 6 bp corresponding to the unique SmaI site used to
produce JEV31 were removed as described above creating JEV33
in which the H6 promoter immediately preceded the ATG start
codon.

     The SmaI-EagI fragment from JEV30 was ligated to SmaI-
EagI digested pTP15 yielding JEV32. The 6 bp corresponding
to the unique SmaI site used to produce JEV32 were removed
as described above creating JEV34 in which the H6 promoter
immediately preceded the ATG start codon. Oligonucleotides
J90 (SEQ ID NO:114), J91 (SEQ ID NO:115), J94 (SEQ ID
NO:116), J95 (SEQ ID NO:117), J96 and J97 (SEQ ID NO:118),
and J99 and J98 (SEQ ID NO:119) are as follows:

```
J90  5'-TCGAG CCCGGG atg ACTAAAAAACCAGGA GGGCC-3'
J91  3'-    C GGGCCC TAC TGATTTTTTGGTCCT C    -5'
         XhoI     SmaI                       ApaI

J94  5'-    C T tga tttttat tga CGGCCG A    -3'
J95  3'-GTACG A ACT AAAAATA ACT GCCGGC TTCGA-5'
        SphI                    EagI   HindIII

J96+J97  5'-GGG atg GGCGTTAACGCACGAGACCGATCAATTGCTTTGGCC
J99+J98  3'-CCC TAC CCGCAATTGCGTGCTCTGGCTAGTTAACGAAACCGG

     TTCTTAGCCACAGGAGGTGTGCTCGTGTTCTTAGCGACCAATGT GCATG-3'
     AAGAATCGGTGTCCTCCACACGAGCACAAGAATCGCTGGTTACA C    -5'
                                                  SphI
```

     Construction of Vaccinia Virus JEV Recombinants.
Plasmids JEV24, JEV27, JEV33 and JEV34 were transfected into
vP410 infected cells to generate the vaccinia recombinants
vP825, vP829, vP857 and vP864 respectively (FIG. 18).

     In Vitro Virus Infection and Radiolabeling. HeLa cell
monolayers were prepared in 35 mm diameter dishes and
infected with vaccinia viruses (m.o.i. of 2 pfu per cell) or
JEV (m.o.i. of 5 pfu per cell) before radiolabeling. Cells

were pulse labeled with medium containing ^{35}S-Met and chased
for 6 hr in the presence of excess unlabeled Met exactly as
described by Mason et al. (1991).

    Radioimmunoprecipitations, Polyacrylamide Gel
Electrophoresis, and Endoglycosidase Treatment.
Radiolabeled cell lysates and culture fluids were harvested
and the viral proteins were immunoprecipitated, digested
with endoglycosidases, and separated in SDS-containing
polyacrylamide gels (SDS-PAGE) exactly as described by Mason
(1989).

    Animal Protection Experiments. Mouse protection
experiments were performed exactly as described by Mason et
al. (1991). Briefly, groups of 3-week-old mice were
immunized by intraperitoneal (ip) injection with 10^7 pfu of
vaccinia virus recombinants, and 3 weeks later sera were
collected from selected mice. Mice were then either re-
inoculated with the recombinant virus or challenged with 1.3
x 10^3 LD_{50} by intraperitoneal injection with a suspension of
suckling mouse brain infected with the P3 strain of JEV.
Three weeks later, the boosted animals were rebled and
challenged with 4.9 x 10^5 LD_{50} of the P3 strain of JEV.
Following challenge, mice were observed at daily intervals
for three weeks and lethal-dose titrations were performed in
each challenge experiment using litter-mates of the
experimental animals. In addition, sera were collected from
all surviving animals 4 weeks after challenge.

    Evaluation of Immune Response to the Recombinant
Vaccinia Viruses. Sera were tested for their ability to
precipitate JEV proteins from detergent-treated cell lysates
or culture fluids obtained from ^{35}S-Met-labeled JEV-infected
cells exactly as described by Mason et al. (1991).
Hemagglutination inhibition (HAI) and neutralization (NEUT)
tests were performed as described by Mason et al. (1991)
except carboxymethylcellulose was used in the overlay medium
for the NEUT test.

    Structure of Recombinant Vaccinia Viruses. Four
different vaccinia recombinants (in the HA locus) were
constructed that expressed portions of the JEV coding region
extending from C through NS2B. The JEV cDNA sequences

contained in these recombinant viruses are shown in FIG. 18.
In all four recombinant viruses the sense strand of the JEV
cDNA was positioned behind the vaccinia virus early/late H6
promoter, and translation was expected to be initiated from
naturally occuring JEV Met codons located at the 5' ends of
the viral cDNA sequences.

Recombinant vP825 encoded the capsid protein,
structural protein precursor prM, the structural
glycoprotein E, the nonstructural glycoprotein NS1, and the
nonstructural protein NS2A (McAda et al., 1987).
Recombinant vP829 encoded the putative 15 aa signal sequence
preceding the amino-terminus of prM, as well as prM, and E
(McAda et al., 1987). Recombinant vP857 contained a cDNA
encoding the 30 aa hydrophobic carboxy-terminus of E,
followed by NS1 and NS2A. Recombinant vP864 contained a
cDNA encoding the same proteins as vP857 with the addition
of NS2B. In recombinants vP825 and vP829 a potential
vaccinia virus early transcription termination signal in E
(TTTTTGT; nucleotides 1304-1310) was modified to TCTTTGT
without altering the aa sequence. This change was made in
an attempt to increase the level of expression of E since
this sequence has been shown to increase transcription
termination in *in vitro* transcription assays (Yuen et al.,
1987).

E and prM Are Correctly Processed When Expressed By
Recombinant Vaccinia Viruses. Pulse-chase experiments
demonstrate that proteins identical in size to E were
synthesized in cells infected with all recombinant vaccinia
viruses containing the E gene (Table 16). In the case of
cells infected with JEV, vP555 and vP829, an E protein that
migrated slower in SDS-PAGE was also detected in the culture
fluid harvested from the infected cells (Table 16). This
extracellular form of E produced by JEV- and vP555-infected
cells contained mature N-linked glycans (Mason, 1989; Mason
et al., 1991), as confirmed for the extracellular forms of E
produced by vP829-infected cells. Interestingly, vP825,
which contained the C coding region in addition to prM and E
specified the synthesis of E in a form that is not released
into the extracellular fluid (Table 16).

Immunoprecipitations prepared from radiolabeled recombinant
vaccinia-infected cells using a MAb specific for M (and prM)
revealed that prM was synthesized in cells infected with
vP555, vP825, and vP829, and M was detected in the culture
fluid of cells infected with vP555 or vP829 (Table 16).

The extracellular fluid harvested from cells infected
with vP555 and vP829 contained an HA activity that was not
detected in the culture fluid of cells infected with vP410,
vP825, vP857 or vP864. This HA appeared similar to the HA
produced in JEV infected cells based on its inhibition by
anti-JEV antibodies and its pH optimum (Mason et al., 1991).
Sucrose density gradients were prepared with culture fluids
from cells infected with vaccinia virus JEV recombinants
vP829, vP825, vP857 and vP864. Analysis of the gradients
identified a peak of HA activity in the vP829 infected
sample that co-migrated with the peak of slowly-sedimenting
hemagglutinin (SHA) found in the JEV culture fluids (data
not shown). This result indicated that vP829 infected cells
produce extracellular particles similar to the empty viral
envelopes containing E and M observed in the culture fluids
harvested from vP555 infected cells (Table 16 and Mason et
al., 1991).

NS1 Is Correctly Processed and Secreted When Expressed
By Recombinant Vaccinia Virus. The results of pulse-chase
experiments demonstrated that proteins identical in size to
authentic NS1 and NS1' were synthesized in cells infected
with vP555, vP825, vP857 and vP864. NS1 produced by vP555-
infected cells was released into the culture fluid of
infected cells in a higher molecular weight form. NS1 was
also released into the culture fluid of cells infected with
vP857 and vP864, whereas NS1 was not released from cells
infected with vP825 (Table 16). Comparison of the synthesis
of NS1 from recombinant vaccinia viruses containing either
the NS2A (vP857) or both the NS2A and NS2B coding regions
showed that the presence or absence of the NS2B coding
region had no affect on NS1 expression, consistent with
previous data showing that only the NS2A gene is needed for
the authentic processing of NS1 (Falgout et al., 1989; Mason
et al., 1991).

Recombinant Vaccinia Viruses Induced Immune Responses
To JEV Antigens. Pre-challenge sera pooled from selected
animals in each group were tested for their ability to
immunoprecipitate radiolabeled E and NS1. The results of
these studies (Table 16) demonstrated that: (1) the
magnitude of immune response induced to E was
vP829>vP555>vP825, (2) all viruses encoding NS1 and NS2A
induced antibodies to NS1, and (3) all immune responses were
increased by a second inoculation with the recombinant
viruses. Analysis of the neutralization and HAI data for
the sera collected from these animals (Table 17) confirmed
the results of the immunoprecipitation analyses, showing
that the immune response to E as demonstrated by RIP
correlated well with these other serological tests (Table
17).

Vaccination With the Recombinant Viruses Provided
Protection From Lethal JEV Infection. All of the
recombinant vaccinia viruses were able to provide mice with
some protection from lethal infection by the peripherally
pathogenic P3 strain of JEV (Huang, 1982) (Table 17). These
studies confirmed the protective potential of vP555 (Mason
et al., 1991) and demonstrated similar protection in animals
inoculated with vP825 and vP829. Recombinant viruses vP857
and vP864 which induced strong immune responses to NS1
showed much lower levels of protection, but mice inoculated
with these recombinants were still significantly protected
when compared to mice inoculated with the control virus,
vP410 (Table 17).

Post-Challenge Immune Responses Document the Level of
JEV Replication. In order to obtain a better understanding
of the mechanism of protection from lethal challenge in
animals inoculated with these recombinant viruses, the
ability of antibodies in post-challenge sera to recognize
JEV antigens was evaluated. For these studies antigen
present in lysates of radiolabeled JEV-infected cells was
utilized, and the response to the NS3 protein which induces
high levels of antibodies in hyperimmunized mice (Mason et
al., 1987a) was examined. The results of these studies
(Table 18) correlates with the survival data in that groups

of animals vaccinated with recombinant viruses that induced
high levels of protection (vP829, vP555, and vP825) showed
low post-challenge responses to NS3, whereas the sera from
survivors of groups vaccinated with recombinants that
expressed NS1 alone (vP857 and vP864) showed much higher
post-challenge responses to NS3.

Table 16. Characterization of proteins expressed by vaccinia recombinants expressing JEV proteins and their immune responses

	vP555	vP829	vP825	vP857	vP864
Proteins expressed[a]					
Intracellular	prM,E NS1	prM,E	prM,E NS1	NS1	NS1
secreted	M,E,NS1	M,E	none	NS1	NS1
Particle formation[b]	+	+	-	-	-
Immune response[c]					
single	E	E	NS1	NS1	NS1
double	E,NS1	E	E,NS1	NS1	NS1

a   Radiolabelled cell lysates and culture fluids from vaccinia virus JEV recombinant infected cells were harvested and JEV-specific proteins immunoprecipitated using mAbs to E, M and NS1 proteins.

b   Formation of extracellular particles with HA activity as described in the text.

c   JEV proteins were immunoprecipitated from radiolabelled JEV-infected cells by sera from mice which had received one (single) or two (double) inoculations of the indicated vaccinia virus JEV recombinant.

Table 17. Protection of mice and immune response following
          single or double inoculations with recombinant
          vaccinia virus expressing JEV proteins

	Immunizing Virus[a]				
Protection[b]	vP555	vP829	vP825	vP857	vP864
single inoculation	7/10	10/10	8/10	0/10	1/10
double inoculation	10/10	9/10	9/10	5/10	6/10
Neut titer[c]					
single inoculation	1:20	1:160	1:10	<1:10	<1:10
double inoculation	1:320	1:2560	1:320	<1:10	<1:10
HAI titer[d]					
single inoculation	1:20	1:40	1:10	<1:10	<1:10
double inoculation	1:80	1:160	1:40	<1:10	<1:10

[a]   Groups of 20 mice were inoculated by ip route with 10^7
      pfu of the indicated vaccinia virus JEV recombinant.
      Sera were collected after three weeks. At this time, 10
      mice per group were challenged with JEV as indicated in
      the text (single inoculation). The remaining 10 mice in
      each group were boosted with the same vaccinia virus
      JEV recombinant (double inoculation). After three
      weeks, sera were collected and the mice were challenged
      with JEV. All mice were observed for 21 days post
      challenge.

[b]   Protection is expressed as number os mice surviving at
      21 days post challenge/total.

[c]   Neutralization titer is expressed as the reciprocal of
      the highest dilution that gives 90% JEV plaque
      reduction.

[d]   HAI titer is expressed as the reciprocal of the highest
      dilution that gives measurable inhibition of
      hemagglutination of red blood cells.

Table 18. Post challenge immune response following single or
         double inoculation with recombinant vaccinia virus
         expressing JEV proteins.

	Immunizing Virus				
	vP555	vP829	vP825	vP857	vP864
single	++	+	++	–a	++++
double	+/–b	–	–	++	+++

+   NS3 antibodies present in post-challenge sera

a   No surviving mice

b   Very low level NS3 antibodies present in post-challenge
    sera

21 day post challenge sera from mice surviving JEV challenge
following single or double inoculation with vaccinia virus
JEV recombinants (Table 17) were analyzed for the presence
of antibodies to JEV NS3.

     Cloning of JEV Genes Into a Vaccinia (NYVAC) Donor
Plasmid. Plasmid pMP2VCL (containing a polylinker region
within vaccinia sequences upstream of the K1L host range
gene) was digested within the polylinker with HindIII and
XhoI and ligated to annealed oligonucleotides SPHPRHA A
through D generating SP126 containing a HindIII site, H6
promoter -124 through -1 (Perkus et al., 1989) and XhoI,
KpnI, SmaI, SacI and EcoRI sites.

     Plasmid pSD544VC (containing vaccinia sequences
surrounding the site of the HA gene which was replaced with
a polylinker region and translation termination codons in
six reading frames) was digested with XhoI within the
polylinker, filled in with the Klenow fragment of DNA
polymerase I and treated with alkaline phosphatase. SP126
was digested with HindIII, treated with Klenow and the H6
promoter isolated by digestion with SmaI. Ligation of the
H6 promoter fragment to pSD544VC generated SPHA-H6 which
contained the H6 promoter in the polylinker region (in the
direction of HA transcription).

     Plasmid JEVL14VC was digested with EcoRV in the H6
promoter and SacI in JEV sequences (nucleotide 2124) and a
1789 bp fragment isolated. JEVL14VC (Mason et al., 1991)
was digested with EclXI at the EagI site following the T5NT,
filled in with the Klenow fragment of DNA polymerase I and
digested with SacI in JEV sequences (nucleotide 2124)
generating a 2005 bp fragment. The 1789 bp EcoRV-SacI and
2005 bp (SacI-filled EclXI) fragments were ligated to EcoRV
(within H6) and SmaI digested (within polylinker) and
alkaline phosphatase treated SPHA-H6 generating JEV35.
JEV35 was transfected into vP866 (NYVAC) infected cells to
generate the vaccinia recombinant vP908 (FIG. 18).

     JEV35 was digested with SacI (within JE sequences
nucleotide 2124) and EclXI (after T5NT) a 5497 bp fragment
isolated and ligated to a SacI (JEV nucleotide 2124) to EagI
fragment of JEV25 (containing the remaining two thirds of E,
translation stop and T5NT) generating JEV36. JEV36 was
transfected into vP866 (NYVAC) infected cells to generate
the vaccinia recombinant vP923 (FIG. 18).

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SPHPRHA A through D Oligonucleotides SPHPRHA:A+B (SEQ ID

NO:120) and SPHPRHA:C+D (SEQ ID NO:121)

are as follows

      HindIII
5'- AGCTTCTTTATTCTATACTTAAAAAGTGAAAATAAATACAAAGGTTCTTGA
3'-   AGAAATAAGATATGAATTTTTCACTTTTATTTATGTTTCCAAGAACT

                                                        EcoRV
GGGTTGTGTTAAATTGAAAGCGAGAAATAATCATAAATTATTTCATTATCGCGATATCCG
CCCAACACAATTTAACTTTCGCTCTTTATTAGTATTTAATAAAGTAATAGCGCTATAGGC

TTAAGTTTGTATCGTAC -3'
AATTCAAACATAGCATGAGCT -5'
                XhoI

    Construction of Plasmids Containing YF Genes. The YF

17D cDNA clones used to construct the YF vaccinia

recombinant viruses (clone 10III and clone 28III), were

obtained from Charles Rice (Washington University School of

Medicine, St. Louis, MO). All nucleotide coordinates are

derived from the sequence data presented in Rice et al.,

1985.

    Plasmid YF0 containing YF cDNA encoding the carboxy-

terminal 80% prM, E and amino-terminal 80% NS1 (nucleotides

537-3266) was derived by cloning an AvaI to NsiI fragment of

YF cDNA (nucleotides 537-1659) and an NsiI to KpnI fragment

of YF cDNA (nucleotides 1660-3266) into AvaI and KpnI

digested IBI25 (International Biotechnologies, Inc., New

Haven, CT). Plasmid YF1 containing YF cDNA encoding C and

amino-terminal 20% prM (nucleotides 119-536) was derived by

cloning a RsaI to AvaI fragment of YF cDNA (nucleotides 166-

536) and annealed oligonucleotides SP46 and SP47 (containing

a disabled HindIII sticky end, XhoI and ClaI sites and YF

nucleotides 119-165) into AvaI and HindIII digested IBI25.

Plasmid YF3 containing YF cDNA encoding the carboxy-terminal

60% of E and amino-terminal 25% of NS1 was generated by

cloning an ApaI to BamHI fragment of YF cDNA (nucleotides

1604-2725) into ApaI and BamHI digested IBI25. Plasmid YF8

containing YF cDNA encoding the carboxy-terminal 20% NS1

NS2a, NS2B and amino-terminal 20% NS3 was derived by cloning

a KpnI to XbaI fragment of YF cDNA (nucleotides 3267-4940)

into KpnI and XbaI digested IBI25. Plasmid YF9 containing

YF cDNA encoding the carboxy-terminal 60% NS2B and amino-

terminal 20% NS3 was generated by cloning a SacI to XbaI
fragment of YF cDNA (nucleotides 4339-4940) into SacI and
XbaI digested IBI25. Plasmid YF13 containing YF cDNA
encoding the carboxy-terminal 25% of C, prM and amino-
terminal 40% of E was derived by cloning a BalI to ApaI
fragment of YF cDNA (nucleotides 384-1603) into ApaI and
SmaI digested IBI25.

     Oligonucleotide-directed mutagenesis (Kunkel, 1985) was
used to change the following potential vaccinia virus early
transcription termination signals (Yuen et al., 1987) (1) 49
aa from the amino-terminus of the C gene in YF1 (TTTTTCT
nucleotides 263-269 and TTTTTGT nucleotides 269-275) to (SEQ
ID NO:122) TTCTTCTTCTTGT creating plasmid YF1B, (2) in the E
gene in YF3 (nucleotides 1886-1893 TTTTTTGT to TTCTTTGT 189
aa from the carboxy-terminus and nucleotides 2429-2435
TTTTTGT to TTCTTGT 8 aa from the carboxy-terminus) creating
plasmids YF3B and YF3C, respectively. A PstI to BamHI
fragment from YF3C (nucleotides 1965-2725) was exchanged for
the corresponding fragment of YF3B generating YF4 containing
YF cDNA encoding the carboxy-terminal 60% E and amino-
terminal 25% NS1 (nucleotides 1604-2725) with both
mutagenized transcription termination signals. An ApaI to
BamHI fragment from YF4 (nucleotides 1604-2725) was
substituted for the equivalent region in YF0 creating
plasmid YF6 containing YF cDNA encoding the carboxy-terminal
80% prM, E and amino-terminal 80% NS1 (nucleotides 537-3266)
with both mutagenized transcription termination signals.
Plasmid YF6 was digested with EcoRV within the IBI25
sequences and AvaI at nucleotide 537 and ligated to an EcoRV
to AvaI fragment from YF1B (EcoRV within IBI25 to AvaI at
nucleotide 536) generating YF2 containing YF cDNA encoding C
through the amino-terminal 80% of NS1 (nucleotides 119-3266)
with an XhoI and ClaI site at 119 and four mutagenized
transcription termination signals.

     Oligonucleotide-directed mutagenesis described above
was used (1) to insert XhoI and ClaI sites preceding the ATG
17 aa from the carboxy-terminus of E (nucleotides 2402-2404)
in plasmid YF3C creating YF5, (2) to insert XhoI and ClaI
sites preceding the ATG 19 aa from the carboxy-terminus of

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prM (nucleotides 917-919) in plasmid YF13 creating YF14, (3)
to insert an XhoI site preceding the ATG 23 aa from the
carboxy-terminus of E (nucleotides 2384-2386) in plasmid
YF3C creating plasmid YF25, (4) and to insert an XhoI site
and ATG (nucleotide 419) in plasmid YF1 21 aa from the
carboxy-terminus of C generating YF45.

     An ApaI to BamHI fragment from YF5 (nucleotides 1604-
2725) was exchanged for the corresponding region of YF0
creating YF7 containing YF cDNA encoding the carboxy-
terminal 80% prM, E and amino-terminal 80% NS1 (nucleotides
537-3266) with XhoI and ClaI sites at 2402 (17 aa from the
carboxy-terminus of E) and a mutagenized transcription
termination signal at 2429-2435 (8 aa from the carboxy-
terminus of E). The ApaI to BamHI fragment from YF25
(nucleotides 1604-2725) was exchanged for the corresponding
region of YF0 generating YF26 containing YF cDNA encoding
the carboxy-terminal 80% prM, E and amino-terminal 80% NS1
(nucleotides 537-3266) with an XhoI site at nucleotide 2384
(23 aa from the carboxy-terminus of E) and mutagenized
transcription termination signal at 2428-2435 (8 aa from the
carboxy-terminus of E).

     An AvaI to ApaI fragment from YF14 (nucleotides 537-
1603) was substituted for the corresponding region in YF6
generating YF15 containing YF cDNA encoding the carboxy-
terminal 80% prM, E and amino-terminal 80% NS1 (nucleotides
537-3266) with XhoI and ClaI sites at nucleotide 917 (19 aa
from the carboxy-terminus of prM) and two mutagenized
transcription termination signals. YF6 was digested within
IBI25 with EcoRV and within YF at nucleotide 537 with AvaI
and ligated to an EcoRV (within IBI25) to AvaI fragment of
YF45 generating YF46 containing YF cDNA encoding C through
the amino-terminal 80% NS1 (nucleotides 119-3266) wit an
XhoI site at 419 (21 aa from the carboxy-terminus of C) and
two transcription termination signals removed.

     Oligonucleotide-directed mutagenesis described above
was used to insert a SmaI site at the carboxy-terminus of
NS2B (nucleotide 4569) in plasmid YF9 creating YF11, and to
insert a SmaI site at the carboxy-terminus of NS2A
(nucleotide 4180) in plasmid YF8 creating YF10. A SacI to

XbaI fragment from YF11 (nucleotides 4339-4940) and Asp718
to SacI fragment from YF8 (nucleotides 3262-4338) were
ligated to Asp718 and XbaI digested IBI25 creating YF12
containing YF cDNA encoding the carboxy-terminal 20% NS1,
NS2A, NS2B and amino-terminal 20% NS2B (nucleotides 3262-
4940) with a SmaI site after the carboxy-terminus of NS2B
(nucleotide 4569).

     Cloning of YF Genes into a pHES System Vaccinia Virus
Donor Plasmid. Prior to insertion of YF cloning sequences
into a NYVAC donor plasmid, YF coding sequences were
inserted into vaccinia plasmid pHES4 (Perkus et al., 1989).
A KpnI to SmaI fragment from YF12 encoding carboxy-terminal
20% NS1, NS2A and NS2B (nucleotides 3267-4569), XhoI to KpnI
fragment from YF15 encoding 19 aa prM, E and amino-terminal
80% NS1 (nucleotides 917-3266) and XhoI-SmaI digested pHES4
were ligated generating YF23. An XhoI to BamHI fragment
from YF26 encoding 23 aa E, amino-terminal 25% NS1
(nucleotides 2384-2725) was ligated to an XhoI to BamHI
fragment from YF23 (containing the carboxy-terminal 75% NS1,
NS2A and NS2B, the origin of replication and vaccinia
sequences) generating YF28.

     XhoI-SmaI digested pHES4 was ligated to a purified XhoI
to KpnI fragment from YF7 encoding 17 aa E and amino-
terminal 80% NS1 (nucleotides 2402-3266) plus a KpnI to SmaI
fragment from YF10 encoding the carboxy-terminal 20% NS1 and
NS2A (nucleotides 3267-4180) creating YF18. An XhoI to
BamHI fragment from YF2 encoding C, prM, E and amino-
terminal 25% NS1 (nucleotides 119-2725) was ligated to a
XhoI to BamHI fragment of YF18 (containing the carboxy-
terminal 75% NS1 and NS2A, the origin of replication and
vaccinia sequences) generating YF19. The same XhoI to BamHI
fragment from YF2 was ligated to a XhoI to BamHI fragment
from YF28 (containing the carboxy-terminal 75% NS1 and NS2A,
the origin of replication and vaccinia sequences) generating
YF20. A XhoI to BamHI fragment from YF46 encoding 21 aa C,
prM, E and amino-terminal 25% NS1 (nucleotides 419-2725) was
ligated to the XhoI to BamHI fragment from YF18 generating
YF47. Oligonucleotide SP46 (SEQ ID NO:123) AND SP47 (SEQ ID
NO:124) are as follows:

          HindIII
    SP46 5'- AGCTT CTCGAGCATCGATTACT atg TCTGGTCGTAAAGCTCAGGG
    SP47 3'-   A GAGCTCGTAGCTAATGA TAC AGACCAGCATTTCGAGTCCC

               AAAAACCCTGGGCGTCAATATGGT -3'
               TTTTTGGGACCCGCAGTTATACCA -5'

        Construction of Recombinant YF Vaccinia Viruses. Five
    different vaccinia virus recombinants that expressed
    portions of the YF coding region extending from C through
    NS2B were constructed utilizing a host range selection
    system (Perkus et al., 1989). Plasmids YF18, YF23, YF20,
    YF19 and YF47 were transfected into vP293 infected cells to
    generate the vaccinia recombinants vP725, vP729, vP764,
    vP766 and vP869. The YF cDNA sequences contained in these
    recombinants are shown in FIG. 19. In all five recombinant
    viruses the sense strand of YF cDNA was positioned behind
    the vaccinia virus early/late H6 promoter, and translation
    was expected to be initiated from Met codons located at the
    5' ends of the viral cDNA sequences (FIG. 19).

        Recombinant vP725 encoded the putative 17-aa signal
    sequence preceding the N terminus of the nonstructural
    protein NS1 and the nonstructural proteins NS1 and NS2A
    (Rice et al., 1985). Recombinant vP729 encoded the putative
    19-aa signal sequence preceding the N terminus of E, E, NS1,
    NS2A and NS2B (Rice et al., 1985). Recombinant vP764
    encoded C, prM, E, NS1, NS2A and NS2B (Rice et al., 1985).
    Recombinant vP766 encoded C, prM, E, NS1 and NS2A (Rice et
    al., 1985). Recombinant vP869 encoded the putative 21-aa
    signal sequence preceding the N terminus of the prM
    structural protein precursor as well as prM, E, NS1 and NS2A
    (Rice et al., 1985).

        Protection From Lethal YF Challenge. vP869 secreted an
    HA activity not found in the culture fluid of cells infected
    with any of the other recombinants. This HA appeared
    similar to the HA produced in YF infected cells based on its
    inhibition by anti-YF antibodies and pH optimum.

        Three-week-old mice were inoculated intraperitoneally
    with 10^7 pfu vP869, vP764 or YF-17D and challenged three
    weeks later with 100 LD_{50} of French neurotropic strain of
    YF. vP869 provided significant protection (Table 19)

whereas vP764 offered no better protection than a control
vaccinia virus lacking YF genes (vP457).

Table 19. Protection of mice by recombinant vaccinia viruses
          from YF challenge

Immunizing Virus   Survival/total

     vP457   2/10
     vP764   2/10
     vP869   9/10
     17D   5/10

     Cloning of YF Genes Into a NYVAC Donor Plasmid. A XhoI
to SmaI fragment from YF47 (nucleotides 419-4180) containing
YF cDNA encoding 21 amino acids C, prM, E, NS1, NS2A (with
nucleotide 2962 missing in NS1) was ligated to XhoI-SmaI
digested SPHA-H6 (HA region donor plasmid) generating YF48.
YF48 was digested with SacI (nucleotide 2490) and partially
digested with Asp718 (nucleotide 3262) and a 6700 bp
fragment isolated (containing the plasmid origin of
replication, vaccinia sequences, 21 amino acids C, prM, E,
amino-terminal 3.5% NS1, carboxy-terminal 23% NS1, NS2A) and
ligated to a SacI-Asp718 fragment from YF18 (containing the
remainder of NS1 with the base present at 2962) generating
YF51. The 6 bp corresponding to the unique XhoI site in
YF51 were removed using oligonucleotide-directed double-
strand break mutagenesis (Mandecki, 1986) creating plasmid
YF50 encoding YF 21 amino acids C, prM, E, NS1, NS2A in the
HA locus donor plasmid. Donor plasmid YF50 was transfected
into vP866 (NYVAC) infected cells to generate vaccinia
recombinant vP984.
     The 6 bp corresponding to the unique XhoI site in YF48
were removed using oligonucleotide-directed double-strand
break mutagenesis creating YF49. Oligonucleotide-directed
mutagenesis (Kunkel, 1985) was used to insert a SmaI site at
the carboxy-terminus of E (nucleotide 2452) in YF4 creating
YF16. An ApaI-SmaI fragment of YF49 (containing the plasmid
origin of replication, vaccinia sequences and YF cDNA
encoding 21 amino acid C, prM, and amino-terminal 43% E) was
ligated to an ApaI-SmaI fragment from YF16 (nucleotides

1604-2452 containing the carboxy-terminal 57% E) generating
YF53 containing 21 amino acids of C, prM, E in the HA locus.
Donor plasmid YF53 was transfected into vP913 (NYVAC-MV)
infected cells to generate the vaccinia recombinant vP997.

Cloning of Dengue Type 1 Into a Vaccinia Virus Donor
Plasmid. Plasmid DEN1 containing DEN cDNA encoding the
carboxy-terminal 84% NS1 and amino-terminal 45% NS2A
(nucleotides 2559-3745, Mason et al., 1987b) was derived by
cloning an EcoRI-XbaI fragment of DEN cDNA (nucleotides
2559-3740) and annealed oligonucleotides DEN1 (SEQ ID
NO:125) and DEN2 (SEQ ID NO:126) (containing a XbaI sticky
end, translation termination codon, T5AT vaccinia virus
early transcription termination signal (Yuen et al. 1987),
EagI site and HindIII sticky end) into HindIII-EcoRI
digested pUC8. An EcoRI-HindIII fragment from DEN1
(nucleotides 2559-3745) and SacI-EcoRI fragment of DEN cDNA
encoding the carboxy-terminal 36% of E and amino-terminal
16% NS1 (nucleotides 1447-2559, Mason et al., 1987b) were
ligated to HindIII -SacI digested IBI24 (International
Biotechnologies, Inc., New Haven, CT) generating DEN3
encoding the carboxy-terminal 64% E through amino-terminal
45% NS2A with a base missing in NS1 (nucleotide 2467).

HindIII-XbaI digested IBI24 was ligated to annealed
oligonucleotides DEN9 (SEQ ID NO:127) and DEN10 (SEQ ID
NO:128) [containing a HindIII sticky end, SmaI site, DEN
nucleotides 377-428 (Mason et al., 1987b) and XbaI sticky
end] generating SPD910. SPD910 was digested with SacI
(within IBI24) and AvaI (within DEN at nucleotide 423) and
ligated to an AvaI-SacI fragment of DEN cDNA (nucleotides
424-1447 Mason et al., 1987) generating DEN4 encoding the
carboxy-terminal 11 aa C, prM and amino-terminal 36% E.

Plasmid DEN6 containing DEN cDNA encoding the carboxy-
terminal 64% E and amino-terminal 18% NS1 (nucleotides 1447-
2579 with nucleotide 2467 present Mason et al., 1987b) was
derived by cloning a SacI-XhoI fragment of DEN cDNA into
IBI25 (International Biotechnologies, Inc., New Haven, CT).
Plasmid DEN15 containing DEN cDNA encoding 51 bases of the
DEN 5' untranslated region, C, prM and amino-terminal 36% E
was derived by cloning a HindIII-SacI fragment of DEN cDNA

(nucleotides 20-1447, Mason et al., 1987b) into HindIII-SacI
digested IBI25. Plasmid DEN23 containing DEN cDNA encoding
the carboxy-terminal 55% NS2A and amino-terminal 28% NS2B
(nucleotides 3745-4213) was derived by cloning a XbaI-SphI
fragment of DEN cDNA into XbaI-SphI digested IBI25. Plasmid
DEN20 containing DEN cDNA encoding the carboxy-terminal 55%
NS2A, NS2B and amino-terminal 24 amino acids NS3
(nucleotides 3745-4563) was derived by cloning a XbaI to
EcoRI fragment of DEN cDNA into XbaI-EcoRI digested IBI25.

     Oligonucleotide-directed mutagenesis (Kunkel, 1985) was
used to change the following potential vaccinia virus early
transcription termination signals (Yuen et al., 1987). The
two T5NT seqeunces in the prM gene in DEN4 were mutagenized
(1) 29 aa from the carboxy-terminus (nucleotides 822-828
TTTTTCT to TATTTCT) and (2) 13 aa from the carboxy-terminus
(nucleotides 870-875 TTTTTAT to TATTTAT) creating plasmid
DEN47. The single T5NT sequence in the NS1 gene in DEN6 17
aa from the amino-terminus was mutagenized (nucleotides
2448-2454 TTTTTGT to TATTTGT) creating plasmid DEN7.

     Oligonucleotide-directed mutagenesis as described above
was used (1) to insert an EagI and EcoRI site at the
carboxy-terminus of NS2A (nucleotide 4102) in plasmid DEN23
creating DEN24, (2) to insert a SmaI site and ATG 15 aa from
the carboxy-terminus of E in DEN7 (nucleotide 2348) creating
DEN10, (3) to insert an EagI and HindIII site at the
carboxy-terminus of NS2B (nucleotide 4492) in plasmid DEN20
creating plasmid DEN21, and (4) to replace nucleotides 63-67
in plasmid DEN15 with part of the vaccinia virus early/late
H6 promoter (positions -1 to -21, Perkus et al., 1989)
creating DEN16 (containing DEN nucleotides 20-59, EcoRV site
to -1 of the H6 promoter and DEN nucleotides 68-1447).

     A SacI-XhoI fragment from DEN7 (nucleotides 1447-2579)
was substituted for the corresponding region in DEN3
generating DEN19 containing DEN cDNA encoding the carboxy-
terminal 64% E and amino-terminal 45% NS2A (nucleotides
1447-3745) with nucleotide 2467 present and the modified
transcription termination signal (nucleotides 2448-2454). A
XhoI-XbaI fragment from DEN19 (nucleotides 2579-3745) and a
XbaI-HindIII fragment from DEN24 (XbaI nucleotide 3745 DEN

through HindIII in IBI25) were ligated to XhoI-HindIII
digested IBI25 creating DEN25 containing DEN cDNA encoding
the carboxy-terminal 82% NS1, NS2A and amino-terminal 28%
NS2B (nucleotides 2579-4213) with a EagI site at 4102,
nucleotide 2467 present and mutagenized transcription
termination signal (nucleotides 2448-2454). The XhoI-XbaI
fragment from DEN19 (nucleotides 2579-3745) was ligated to
XhoI (within IBI25) and XbaI (DEN nucleotide 3745) digested
DEN21 creating DEN22 encoding the carboxy-terminal 82% NS1,
NS2A, NS2B and amino-terminal 24 aa NS3 (nucleotides 2579-
4564) with nucleotide 2467 present, modified transcription
termination signal (nucleotides 2448-2454) and EagI site at
4492.

     A HindIII-PstI fragment of DEN16 (nucleotides 20-494)
was ligated to a HindIII-PstI fragment from DEN47 (encoding
the carboxy-terminal 83% prM and amino-terminal 36% of E
nucleotides 494-1447 and plasmid origin of replication)
generating DEN17 encoding C, prM and amino-terminal 36% E
with part of the H6 promoter and EcoRV site preceding the
amino-terminus of C. A HindIII-BglII fragment from DEN17
encoding the carboxy-terminal 13 aa C, prM and amino-
terminal 36% E (nucleotides 370-1447) was ligated to
annealed oligonucleotides SP111 and SP112 (containing a
disabled HindIII sticky end, EcoRV site to -1 of the H6
promoter, and DEN nucleotides 350-369 with a BglII sticky
end) creating DEN33 encoding the EcoRV site to -1 of the H6
promoter, carboxy-terminal 20 aa C, prM and amino-terminal
36% E.

     SmaI-EagI digested pTP15 (Mason et al., 1991) was
ligated to a SmaI-SacI fragment from DEN4 encoding the
carboxy-terminal 11 aa C, prM and amino-terminal 36% E
(nucleotides 377-1447) and SacI-EagI fragment from DEN3
encoding the carboxy-terminal 64% E, NS1 and amino-terminal
45% NS2A generating DENL. The SacI-XhoI fragment from DEN7
encoding the carboxy-terminal 64% E and amino-terminal 18%
NS1 (nucleotides 1447-2579) was ligated to a BstEII-SacI
fragment from DEN47 (encoding the carboxy-terminal 55% prM
and amino-terminal 36% E nucleotides 631-1447) and a BstEII-
XhoI fragment from DENL (containing the carboxy-terminal 11

aa C, amino-terminal 45% prM, carboxy-terminal 82% NS1,
carboxy-terminal 45% NS2A, origin of replication and
vaccinia sequences) generating DEN8. A unique _SmaI_ site
(located between the H6 promoter and ATG) was removed using
oligonucleotide-directed double-strand break mutagenesis
(Mandecki, 1986) creating DEN8VC in which the H6 promoter
immediately preceded the ATG start codon.

An _EcoRV-SacI_ fragment from DEN17 (positions -21 to -1
H6 promoter DEN nucleotides 68-1447) was ligated to an _EcoRV_
-_SacI_ fragment of DEN8VC (containing vaccinia sequences, H6
promoter from -21 to -124, origin of replication and amino-
terminal 64% E, NS1, amino-terminal 45% NS2A nucleotides
1447-3745) generating DEN18. A _XhoI-EagI_ fragment from
DEN25 (nucleotides 2579-4102) was ligated to an _XhoI-EagI_
fragment of DEN18 (containing the origin of replication,
vaccinia sequences and DEN C, prM, E and amino-terminal 18%
NS1 nucleotides 68-2579) generating DEN26. An _EcoRV-SacI_
fragment from DEN8VC (positions -21 to -1 H6 promoter DEN
nucleotides 377-1447) was ligated to an _EcoRV-SacI_ fragment
of DEN26 (containing the origin of replication, vaccinia
sequences and DEN region encoding the carboxy-terminal 64%
E, NS1 and NS2A with a base missing in NS1 at nucleotide
2894) generating DEN32. DEN32 was transfected into vP410
infected cells to generate the recombinant vP867 (FIG. 20).

A _SacI-XhoI_ fragment from DEN10 (nucleotides 1447-2579)
was substituted for the corresponding region in DEN3
generating DEN11 containing DEN cDNA encoding the carboxy-
terminal 64% E, NS1 and amino-terminal 45% NS2A with a _SmaI_
site and ATG 15 aa from the carboxy-terminus of E. A _SmaI-_
EagI fragment from DEN11 (encoding the carboxy-terminal 15
aa E, NS1 and amino-terminal 45% NS2A nucleotides 2348-3745)
was ligated to _SmaI-EagI_ digested pTP15 generating DEN12.

A _XhoI-EagI_ fragment from DEN22 (nucleotides 2579-4492)
was ligated to the _XhoI-EagI_ fragment from DEN18 described
above generating DEN27. An _EcoRV-PstI_ fragment from DEN12
(positions -21 to -1 H6 promoter DEN nucleotides 2348-3447)
was ligated to an _EcoRV-PstI_ fragment from DEN27 (containing
the origin of replication, vaccinia sequences, H6 promoter -

21 to -124 and DEN cDNA encoding NS2A and NS2B) generating
DEN31.

An EcoRV-XhoI fragment from DEN8VC (positions -21 to -1
H6 promoter DEN nucleotides 377-2579 encoding the carboxy-
terminal 11 aa C, prM E, amino-terminal 18% NS1) was ligated
to an EcoRV-XhoI fragment from DEN31 (containing the origin
of replication, vaccinia sequences and DEN cDNA encoding the
carboxy-terminal 82% NS1, NS2A, NS2B with the base present
in NS1 at position 2894) generating DEN35. DEN35 was
transfected into vP410 infected cells generating the
recombinant vP955 (FIG. 20). An EcoRV-SacI fragment from
DEN33 (positions -21 to -1 H6 promoter DEN nucleotides 350-
1447 encoding the carboxy-terminal 20 aa C, prM and amino-
terminal 36% E) and a SacI-XhoI fragment from DEN32
(encoding the carboxy-terminal 64% E and amino-terminal 18%
NS1 nucleotides 1447-2579) were ligated to the EcoRV-SacI
fragment from DEN31 described above generating DEN34. DEN34
was transfected into vP410 infected cells generating the
recombinant vP962 (FIG. 20). Oligonucleotides DEN 1 (SEQ ID
NO:125), DEN 2 (SEQ ID NO:126), DEN9 (SEQ ID NO:127), DEN10
(SEQ ID NO:128), SP111 (SEQ ID NO:129), and SP112 (SEQ ID
NO:130) are as follows:

```
DEN1  5'-  CTAGA tga TTTTTAT CGGCCG A      -3'
DEN2  3'-      T ACT AAAAATA GCCGGC TTCGA -5'
             XbaI                  EagI    HindIII

DEN9  5'   AGCTT CCCGGG atg CTCCTCATGCTGCTGCCC
DEN10 3'       A GGGCCC TAC GAGGAGTACGACGACGGG
          HindIII  SmaI

            ACAGCCCTGGCGTTCCATCTGACCACCCGAG T      -3'
            TGTCGGGACCGCAAGGTAGACTGGTGGGCTC AGATC  -5'
                                          AvaI     XbaI

            -24         H6         -1
SP111 5' AGCT GATATCCGTTAAGTTTGTATCGTA atg AACAGGA
SP112 3'    A CTATAGGCAATTCAAACATAGCAT TAC TTGTCCT
         HindIII EcoRV
                                      GGAAA A      -3'
                                      CCTTT TCTAG-5'
                                            BglII
```

Example 17 - CONSTRUCTION OF MODIFIED NYVAC VIRUSES

NYVAC was modified by increasing to different extents
the size of the [C7L - K1L] deletion near the left terminus
of vaccinia and by introducing a deletion near the right

terminus. All deletions were accomplished using the *E. coli* guanine phosphoribosyl transferase gene and mycophenolic acid in a transient selection system.

Transient Dominant Selection. Using circular donor plasmid, recombination with vaccinia virus was performed by the standard method of transfection of calcium phosphate precipitated plasmid DNA into vaccinia-infected Vero cells. After 24 h, the infected cells were harvested and the lysate plated in the presence of 1 microgram/ml mycophenolic acid (MPA). Individual plaques were picked and amplified on Vero cells in the presence of MPA. Virus was harvested and plaque purified by two rounds of plaque picking in the absence of MPA. Plaques picked from each round without MPA were plated on Vero cells and filters hybridized for the presence of pertinent genes.

NYVAC.1. The [C7L - K1L] deletion present in vP866 (NYVAC) was expanded to include the next two ORFs to the right, K2L and K3L. The putative translation product for the K2L ORF shows homology to the family of serine protease inhibitors (Boursnell et al., 1988). However, transcriptional mapping of this region of the vaccinia genome suggests that the K2L ORF is not expressed (Morgan et al., 1984).

The translation product for K3L shows 28 % homology to eukaryotic initiation factor 2 alpha (eIF-2 alpha) over an 87 amino acid overlap spanning the serine (amino acid 51) phosphorylation site. Phosphorylation of eIF-2 alpha is a step in the antiviral state induced by interferon, suggesting that the vaccinia K3L gene product may be involved in the mechanism by which vaccinia evades the effects of interferon. The K3L gene from Copenhagen strain of vaccinia has been deleted (Beattie et al., 1991). The resulting virus exhibited heightened sensitivity to interferon *in vitro* as measured both by inhibition of viral induction of protein synthesis and inhibition of viral replication. This suggests that deletion of K3L from vaccinia virus could result in a safer vaccine strain which could be controlled by interferon treatment in the event of vaccination complications.

Construction of Plasmid pMPC7K3GPT for Deletion of C7L
Through K3L. The left and right vaccinia arms flanking the
[C7L - K3L] deletion were assembled separately. The left
arm was derived from pSD420 (Perkus et al., 1990) and
assembled in intermediate deletion plasmid pMP256/257
(Perkus et al., 1991). Synthetic oligonucleotides MPSYN379
(SEQ ID NO:131), MPSYN380 (SEQ ID NO:132)

```
                HindIII SalI          BamHI
MPSYN379 5' TTCCCAAGCTTGTCGACGATAATATGGATCCTCATGAC 3'

                BglII
MPSYN380 5' TTCCCAGATCTATGAGTATAGTGTTAAATGAC 3'
```

were used as primers in a PCR reaction using plasmid pSD420
as template. The resulting 0.14 kb fragment was cut with
HindIII/BglII and inserted into pMP256/257, replacing the
left arm of the plasmid. The resulting plasmid was
designated pMP379/380. A 0.7 kb SalI/BamHI fragment was
isolated from pSD420 and ligated into pMP379/380 cut with
SalI/BamHI, forming plasmid pMPC7F4.

To construct a right deletion junction containing
sequences to the right of K3L, synthetic oligonucleotides
MPSYN381/MPSYN382 (SEQ ID NO:133/SEQ ID NO:134)

```
            BamHI    HpaI     EcoRV SmaI EcoRI
MPSYN381 5' GATCCTTGTTAACCCGATATCCCGGG     3'
MPSYN382 3'     GAACAATTGGGCTATAGGGCCCTTAA 5'
```

were annealed and ligated into pUC8 cut with BamHI/EcoRI,
forming plasmid pMP381/382. A 1.0 kb HpaI (partial) /EcoRV
fragment was isolated from cloned vaccinia HindIII K and
ligated into pMP381/382, forming plasmid pMPK3R, which
contains the entire right vaccinia flanking arm. The left
vaccinia flanking arm was isolated from pMPC7F4 as a 0.8 kb
BglII(partial)/HindIII fragment, and inserted into pMPK3R
cut with BamHI/HindIII. The resulting plasmid, pMPC7K3, is
deleted for 14 genes [C7L - K3L].

For use as a selectable marker, the E. coli gene
encoding guanine phosphoribosyl transferase (Ecogpt) (Pratt
et al., 1983) was placed under the control of a poxvirus

promoter. A 31 bp promoter element immediately upstream
from a gene encoding an entomopox 42 kDa protein can
function as a strong promoter in recombinant vaccinia virus
at early time post infection. Annealed synthetic
oligonucleotides MPSYN369/370 (SEQ ID NO:135/SEQ ID NO:136)

```
                XhoI EcoRI SmaI
MPSYN369 5' TCGAGAATTCCCGGGTCAAAATTGAAAATATATAATTACAA

                                              BglII
                                       TATAAAATA  3'

MPSYN370 3'   CTTAAGGGCCCAGTTTTAACTTTTATATATTAATGTTA

                                       TATTTTATCTAG 5'
```

containing the 31 bp EPV 42 kDa promoter were ligated
upstream from the Ecogpt gene in a pBS-SK background,
resulting in plasmid pMP42GPT. A SmaI fragment containing
the 42 kDa promoter/Ecogpt gene expression cassette was
isolated from pMP42GPT and inserted into vaccinia deletion
plasmid pMPC7K3 in the SmaI site at the pUC/vaccinia
junction. The resulting plasmid, pMPC7K3GPT was transfected
into vP866 (NYVAC). Mycophenolic acid was used in the
culture medium for selection of intermediate products of
recombination in a transient dominant selection system
(Falkner et al., 1990). After removal of selective
pressure, progeny virus were screened by plaque
hybridization for loss of K2L DNA sequences and retention of
K4L. The fidelity of the deletion junction was verified by
PCR and DNA restriction and sequence analysis. Recombinant
vaccinia virus vP954 (NYVAC.1) contains the [C7L - K3L]
deletion, as well the other deletions present in NYVAC (TK,
HA, ATI, I4L, [B13 - B14]).

    NYVAC.2. The [C7L - K1L] deletion present in NYVAC was
expanded in both directions to include a total of 38 ORFs,
[C23L - F4L]. This is the same deletion previously reported
in vaccinia deletion mutant vP796 (Perkus et al., 1991).
Noteworthy ORFs removed in the expanded deletion region
include the vaccinia growth factor (VGF; C11R) located to
the left of the NYVAC deletion. In contrast to WR strain of
vaccinia which contains two copies of the VGF, C11R is the
only ORF encoding the VGF in Copenhagen strain of vaccinia.
Deletion of both copies of the vaccinia growth factor from

WR has been shown to reduce the severity of skin lesions
upon intradermal inoculation of rabbits and to reduce
neurovirulence of the virus in mice (Buller et al., 1988).
The rightmost ORF in the [C23L - F4L] deletion, F4L, encodes
the gene for the small subunit of ribonucleotide reductase
(Slabaugh et al., 1988). Also included in this deletion is
ORF F2L, which shows homology to *E. coli* dUTPase, another
enzyme involved in nucleotide metabolism (Goebel et al.,
1990a,b). F2L also shows homology to retroviral protease
(Slabaugh et al., 1989).

     Construction of Plasmid pMPTRF4GPT for Deletion of C23L
Through F4L. Plasmid pMPLENDA, which was used as an
intermediate in the generation of vaccinia deletion mutant
vP796 (Perkus et al., 1991) was modified by the addition of
the SmaI expression cassette containing the EPV 42 kDa
promoter/Ecogpt gene at the pUC/vaccinia junction. The
resulting plasmid, pMPTRF4GPT, was transfected into NYVAC.
Following selection using MPA, progeny virus were screened
by plaque hybridization for loss of F4L DNA sequences and
retention of F5L. Fidelity of the deletion junction was
verified by PCR and DNA restriction analysis. Recombinant
virus vP938 (NYVAC.2) contains the [C23L - F4L] deletion as
well as the other deletions present in NYVAC.

     Deletion of ORFs B13R - B29R. The u deletion [B13R -
B14R] present in NYVAC was expanded to include all ORFs to
the right, a total of 17 ORFs [B13R - B29R]. This is the
same deletion previously reported in vaccinia deletion
mutant vP759 (Perkus et al., 1991). The expanded deletion
region includes two genes whose products show 20 % amino
acid identity with each other (Smith et al., 1991). The
ORFs encoding these gene products are designated B16R and
B19R in Copenhagen (Goebel et al., 1990a,b), which
correspond to ORFs B15R and B18R, respectively, in the WR
strain (Smith et al., 1991). Unlike the WR strain of
vaccinia, in which both gene products contain typical signal
sequences, the predicted translation product of Copenhagen
ORF B16 is truncated at the amino terminus and does not
contain a signal sequence. B19R encodes a vaccinia surface
protein (S antigen) expressed at early times post infection

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(Ueda et al., 1990). Both B16R and B19R show homology to
the immunoglobin superfamily, especially the IL-1 receptor.
It has been suggested that one or both of the vaccinia gene
products may help vaccinia evade the immune system by
binding cytokines and thus diminishing the host inflammatory
response (Smith et al., 1991).

    Construction of Plasmid pMPTRB13GPT for Deletion of
B13R Through B29R. Plasmid pMPRENDΔ, which was used as an
intermediate in the generation of vaccinia deletion mutants
vP759 and vP811 (Perkus et al., 1991) was modified by the
addition of the SmaI expression cassette containing the EPV
42 kDa promoter/Ecogpt gene at the pUC/vaccinia junction.
The resulting plasmid, pMPTRB13GPT, was transfected into
NYVAC. Following selection using MPA, progeny virus were
screened by plaque hybridization for loss of B15 DNA
sequences and retention of B12. Fidelity of the deletion
junction was verified by PCR and DNA restriction analysis.
Recombinant virus vP953 contains the [B13R - B29R] deletion
as well as the other deletions present in NYVAC.

    Combining the Left [C23L - F4L] and Right [B13R - B29R]
Deletions. The generation of vaccinia deletion mutant
vP811, which contains deletions at both the left [C23L -
F4L] and right [B13R - B29R] termini of vaccinia virus has
been described (Perkus et al., 1991). vP811 contains both
the vaccinia host range gene, C7L, and the selectable marker
Ecogpt. To generate a virus containing the large terminal
deletions without C7L or Ecogpt in a NYVAC background,
pMPTRF4GPT was used as donor plasmid for recombination with
vP953. Progeny virus is being selected by MPA in the
transient dominant selection system described above and
screened by plaque hybridization for loss of F4L DNA
sequences and retention of F5L. Recombinant virus vP977
contains deletions for [B13R-B29R] and [C23L-F4L] as well as
the other deletions present in NYVAC. Like vP811, vP977 is
deleted for all genes from both copies of the vaccinia
terminal repeats.

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**Example 18 - EXPRESSION OF HIV GENE PRODUCTS BY
            HOST-RESTRICTED POXVIRUSES**

This Example describes the generation of host-
restricted poxviruses that express HIV-1 gene products. The
vectors employed are NYVAC and ALVAC.

Cells and Virus. NYVAC and ALVAC viral vectors and
their derivatives were propagated as described previously
(Piccini et al., 1989; Taylor et al., 1988a, b). Vero cells
and primary chick embryo fibroblasts (CEF) were propagated
as described previously (Taylor et al., 1988a, b). P815
murine mastocytoma cells (H-2^d) were obtained from ATCC
(#TIB64) and maintained in Eagles MEM supplemented with 10%
fetal bovine serum CFBS and 100 Iu/ml penicillin and 100 μg
streptomycin per ml.

Mice. Female BALB/cJ (H-2^d) mice were purchased from
Jackson Laboratories (Bar Harbor, ME) and maintained on
mouse chow and water ad libitum. All mice were used between
the ages of 6 and 15 weeks of age.

Media. Assay Medium for immunological assays was
comprised of RPMI 1640 medium supplemented with 10% FBS, 4
mM L-glutamine, 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-
2-ethanesulfonate), 5x10^{-5} M 2-mercaptoethanol, 100 IU
penicillin per ml, and 100 μg/ml streptomycin. Stim Medium
was comprised of Eagle's Minimum Essential Medium
supplemented with 10% FBS, 4 mM L-glutamine, 10^{-4} M
2-mercaptoethanol, 100 IU penicillin per ml, and 100 μg
streptomycin per ml.

_ALVAC and NYVAC Recombinants Containing the V3 Loop and
Epitope 88 of the HIV-1 (IIIB) Envelope._ A 150 bp fragment
encompassing the V3 loop (amino acids 299-344; Javeherian et
al., 1989) of HIV-1 (IIIB) was derived by PCR using
oligonucleotides HIV3BL5 (SEQ ID NO:137) (5'-ATGGTAGAAA
TTAATTGTAC-3') and HIV3BL3 (SEQ ID NO:138) (5'-ATCATCGAATTCA
AGCTTATTATTTTGCTCTACTAATGTTAC-3') with pHXB.2D (III) as
template (provided by Dr. R. C. Gallo, NCI-NIH, Bethesda,
MD). Oligonucleotides HIV88A (SEQ ID NO:139) (5'-
ATGAATGTGACAGAAAATTTTAACATGTGGAAAAATGTAGAAATTAATTGTACAAGACCC
-3') and HIV88B (SEQ ID NO:140) (5'-
GGGTCTTGTACAATTAATTTCTACATTTTTCCACATGTTAAAATTTTCTGTCACATTCAT

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-3') were annealed together to produce a double-stranded
fragment containing the HIV-1 epitope 88 (amino acids 95-
105, Shaffermann et al., 1989). The 150 bp V3-containing
PCR fragment containing the epitope and the 42 bp fragment
containing the 88 epitope sequences were fused together by
PCR by virtue of the existence of complementary sequences.
The reactions were performed using oligonucleotides HIV88C
(SEQ ID NO:141) (5'-AGTAATGTGACAGAAAATTTTAAC-3') and HIV3BL3
(SEQ ID NO:138). The 192 bp PCR-derived fragment contains
the epitope 88 sequences fused upstream to the V3 loop
sequences. A termination codon (TAA) was incorporated into
oligonucleotide HIV3BL3P to terminate translation of the
open reading frame and an initiation codon was incorporated
into oligonucleotide HIV88C to serve as the start of
translation to express the epitope 88/V3 loop fusion
protein. Additionally, oligonucleotide HIV3BL3 was
synthesized so that an EcoRI site existed at the 3'-end of
the 192 bp PCR fragment.

The entomopoxvirus 42 kDa (early) promoter was
generated by PCR using oligonucleotides RG273 (SEQ ID
NO:142) (5'-AGGCAAGCTTTCAAAAAAATATAAATGATTC-3') and RG274
(SEQ ID NO:143) (5'-TTTATATTGTAATTATATATTTTC-3') with
plasmid, pAM12, as template. The 108 bp fragment containing
the 42 kDa promoter was synthesized to contain a HindIII
site at the 5'-end. The 42 kDa promoter containing segment
was kinased and digested with HindIII prior to ligation to
the epitope 88/V3 fragment digested with EcoRI and pRW831
digested with HindIII and EcoRI. The resultant plasmid was
designated as pC5HIVL88. This plasmid was used in in vitro
recombination assays with CPpp as rescue virus to generate
vCP95. ALVAC recombinant, vCP95, contains the epitope 88/V3
loop in the de-ORFed C5 locus of CPpp.

The plasmid pC5HIVL88 was digested with HindIII and
EcoRI to liberate a 300 bp fragment containing the epitope
88/V3 expression cassette described above. This fragment
was excised from a LMP-agarose gel and isolated by phenol
extraction (2X) and ether extraction (1X). The isolated
fragment was blunt-ended using the Klenow fragment of the E.
coli DNA polymerase in the presence of 2mM dNTPs. The

fragment was ligated to pSD550, a derivative of pSD548 (FIG.
6) digested with SmaI to yield plasmid pHIVL88VC. This
plasmid was used with vP866 as the rescue virus to generate
vP878. vP878 contains the epitope 88/V3 loop cassette in
the de-ORFed I4L locus of NYVAC.

ALVAC- and NYVAC-Based Recombinants Expressing the HIV-
1 (IIIB) Envelope Glycoproteins. An expression cassette
composed of the HIV-1 (IIIB) env gene juxtaposed 3' to the
vaccinia virus H6 promoter (Guo et al., 1989; Taylor et al.,
1988a, b) was engineered for expression of gp160 from HIV-1
by the ALVAC and NYVAC vectors. A 1.4 kb fragment was
amplified from pHXB.2D (III) (provided by Dr. R.C. Gallo,
NCI-NIH, Bethesda, MD) using oligonucleotides HIV3B1 (SEQ ID
NO:144) (5'-GTTTTAATTGTGGAGGGGAATTCTTCTACTGTAATTC-3') and
HIV3B5 (SEQ ID NO:145) (5'-
ATCATCTCTAGAATAAAAATTATAGCAAAATCCTTTC-3'). This fragment
contains the 3' portion of the env gene. PCR amplification
with these primers placed a vaccinia virus early
transcription termination T5NT sequence motif following the
coding sequence and removed the T5NT motif situated at
position 6146 to 6152 (Ratner et al., 1985) without altering
the amino acid sequence. This change (T to C) creates an
EcoRI (GAATTC) at this position. This 1.4 kb fragment was
digested with EcoRI (5'- end) and XbaI (3'- end) and
inserted into EcoRI and XbaI digested pBS-SK (Stragagene, La
Jolla, CA). The resultant plasmid was designated as
pBSHIVENV1,5. Nucleotide sequence analysis of this fragment
demonstrated that the sequence was entirely correct except
for a T to C transition at position 7848. This transition
was corrected as follows: A 250 bp fragment was derived by
PCR using oligonucleotides HIV3B1 (SEQ ID NO:144) (5'-
GTTTTAATTGTGGAGGGGAATTCTTCTACTGTAATTC-3') and HIV3B17 (SEQ
ID NO:146) (5'-TGCTACTCCTAATGGTTC-3') with pHXB.2D (III) as
template. This fragment was digested with BglII and EcoRI.
The fragment was inserted into pBSHIV3B1,5, digested with
BglII and EcoRI and thus substituted for the region with the
incorrect nucleotide to yield plasmid pBSHIV3BBP.

PCR was utilized to derive a 150 bp fragment containing
the 5' portion of the env gene with oligonucleotides HIV3B9

(SEQ ID NO:147) (5'-CATATGCTTTAGCATCTGATG-3') and HIV3B10
(SEQ ID NO:148) (5'-ATGAAAGAGCAGAAGACAGTG-3') with pHXB.2D
(III) as template. PCR was also used to generate a 128 bp
fragment containing the vaccinia virus H6 promoter from
pC3FGAG using oligonucleotides VVH65P (SEQ ID NO:149) (5'-
ATCATCGGTACCGATTCTTTATTCTATAC-3') and VVH63P (SEQ ID NO:150)
(5'-TACGATACAAACTTAACGG-3'). Both fragments were digested
with KpnI and the 150 bp fragment was kinased prior to co-
insertion of these fragments into pBS-SK digested with KpnI.
The resultant plasmid was designated as pBSH6HIV3B5P.

     PCR was used to generate a 600 bp fragment from pHXB.2D
(III) with oligonucleotides HIV3B2 (SEQ ID NO:151) (5'-
GAATTACAGTAGAAGAATTCCCCTCCACAATTAAAAC-3') and HIV3B7 (SEQ ID
NO:152) (5'-CAATAGATAATGATACTAC-3'). This fragment was
digested with EcoRI and kinased. PCR was also used to
derive a 500 bp fragment with the same template but with
oligonucleotides HIV3B6 (SEQ ID NO:153) (5'-
GTATTATATCAAGTTTATATAATAATGCATATTC-3') and HIV3B8 (SEQ ID
NO:154) (5'-GTTGATGATCTGTAGTGC-3'). This fragment was
digested with KpnI. These fragments together correspond to
nucleotide 5878 to 6368 (Ratner et al., 1985). The
engineering of these fragments with these primers also
removes a T5NT sequence positioned at nucleotide 6322 to
6328 without altering the amino acid sequence. These two
fragments were inserted into pBSHIV3B3P digested with KpnI
and EcoRI. This plasmid was designated as pBSHIV3BP2768.

     Plasmid pBSH6HIV3B5P was digested with KpnI to liberate
a 360 bp fragment containing the H6 promoter and the 5'
portion (150 bp) of the HIV-1 env gene. This KpnI fragment
was ligated into pBSHIV3B3P2768 digested with KpnI to yield
plasmid pBSHIV3BEAII. A 2.8 kb fragment was derived from
pBSHIV3BEAII by digestion with XbaI followed by a partial
KpnI digestion. This fragment was blunt-ended and inserted
into SmaI digested pSD550. The plasmid pI4LH6HIV3B was
generated and used in in vitro recombination experiments
with vP866 as the rescue virus. This generated vP911 which
contains the HIV-1 env gene in the I4L locus of the NYVAC
genome.

     To insert the HIV-1 env gene into an ALVAC vector,
pBSHIV3BEAII was digested with NruI and XbaI. The derived
2.7 kb fragment was blunt-ended with the Klenow fragment of
the E. coli DNA polymerase in the presence of 2mM dNTPs.
This fragment contains the entire HIV-1 env gene juxtaposed
3' to the 3'-most 21 bp (to NruI site) of the vaccinia H6
promoter. This fragment was ligated to a 3.1 kb fragment
derived by digestion of pRW838 with NruI and EcoRI with
subsequent blunt-ending with Klenow. The pRW838 derived
fragment contains the homologous arms derived from canarypox
to direct the foreign gene to the C5 locus. It also
contains the 5'-most 100 bp of the H6 promoter. Therefore,
ligation of these fragments resulted in an insertion plasmid
containing an expression cassette for the HIV-1 env gene and
was designated pC5HIV3BE. This plasmid was used in in vitro
recombination experiments with ALVAC as the rescue virus to
generate vCP112.

     NYVAC-Based Recombinants Expressing the HIV-1 (IIIB)
gp120. The plasmid pBSHIV3BEAII was digested with EcoRI and
XbaI to liberate a 4.3 kb fragment. This fragment contains
the vaccinia virus H6 promoter linked to the HIV-1 env gene
to nucleotide 6946 (Ratner et al., 1985). The 4.3 kb
fragment was ligated to 300 bp EcoRI/XbaI digested PCR-
derived fragment corresponding to the 3' portion of the
gp120 coding sequence. The 300 bp PCR fragment was derived
using oligonucleotides HIV1-120A (SEQ ID NO:155) (5'
-ATCATCTCTAGAATAAAAATTATGGTTCAATTTTTACTACTTTTATATTATATATTTC-
3') and HIV1-120B (SEQ ID NO:156) (5'-
CAATAATCTTTAAGCAAATCCTC-3') with pHXB.2D as template. The
ligation of the 4.3 kb XbaI/EcoRI fragment and the 300 bp
XbaI/EcoRI fragment yielded plasmid pBSHIVB120.

     A 1.6 kb KpnI/XbaI fragment was derived from pBSHIVB120
by initially linearizing the plasmid with XbaI followed by a
partial KpnI digestion. The 1.6 kb fragment was blunt-ended
by treatment with the Klenow fragment of the E. coli DNA
polymerase in the presence of 2mM dNTPs. This fragment was
inserted into pSD54IVC digested with SmaI to yield
pATIHIVB120. This plasmid was used in in vitro
recombination experiments to generate vP921. This

recombinant contains the portion of the HIV-1 env gene
encoding gp120 in the ATI locus of NYVAC.

Immunoprecipitation. To determine the authenticity of
the HIV-1 gene products expressed by vP911, vP921 and
vCP112, immunoprecipitation analyses were performed. Vero
cells monolayers were either mock infected, infected with
the parental virus vP866, or infected with recombinant virus
at an m.o.i. of 10 PFU/cell. Following a 1 hr adsorption
period, the inoculum was aspirated and the cells were
overlayed with 2 mls of MEM (minus methionine containing 2%
FBS and [^{35}S]-methionine (20 uCi/ml). Cells were harvested
at 18 hr post infection by the addition of 1 ml of 3X Buffer
A (3% NP-40, 30mM Tris pH 7.4, 150 mM NaCl, 3mM EDTA, 0.03%
Na Azide, and 0.6 mg/ml PMSF) with subsequent scraping of
the cell monolayers.

Lysates derived from the infected cells were analyzed
for HIV-1 env gene expression using pooled serum from HIV-1
seropositive individuals (obtained from Dr. Genoveffa
Franchini NCI-NIH, Bethesda MD). The sera was preadsorbed
with vP866-infected Vero cells. The preadsorbed human sera
was bound to protein A-sepharose in an overnight incubation
at 4°C. In some cases a monoclonal antiserum specific to
gp120 (Dupont) was used as the primary serum and a rat anti-
mouse as the second antibody. Following this incubation
period, the material was washed 4 times with 1X Buffer A.
Lysates precleared with normal human sera and protein A-
Sepharose were then incubated overnight at 4°C with the
human sera from seropositive individuals bound to protein A-
Sepharose. Following the overnight incubation period, the
samples were washed four times with 1X Buffer A and 2X with
LiCl$_2$/urea buffer. Precipitated proteins were dissociated
from the immune complexes by the addition of 2X Laemmli's
buffer (125 mM Tris (pH6.8), 4% SDS, 20% glycerol; 10% 2-
mercaptoethanol) and boiling for 5 minutes. Proteins were
fractionated on a 10% Dreyfuss gel system (Dreyfuss et al.,
1984), fixed and treated with 1M Na - salicylate for
fluorography.

The results of immunoprecipitation using sera pooled
from HIV-1 seropositive individuals showed specific

precipitation of the gp120 and gp41 mature forms of the
gp160 envelope glycoprotein from vP911 infected cell
lysates. No such specific gene products were detected in
the parentally (NYVAC; vP866) infected cell lysates.
Specific precipitation of gp120 was also found in vP921
infected cell lysates.

Immunofluorescence analysis with the same sera
illustrated that the gp160 and gp120 species expressed by
vP911 and vP921, respectively, were present on the surface
of infected cells.

Immunoprecipitation was also performed with vCP112
infected CEF cells. No HIV-specific polypeptides were
precipitated with a monoclonal antibody directed against the
gp120 extracellular moiety from cells infected with the
ALVAC parental virus and uninfected CEF cells. Two HIV-
specific polypeptides species were, however, precipitated
from vCP112 infected cells. These species migrated with
apparent mobilities of 160 kDa and 120 kDa, corresponding to
the precursor env gene product and the mature extracellular
form, respectively.

Inoculations. Mice were intravenously inoculated with
5×10^7 plaque forming units (PFU) in 0.1 ml of phosphate-
buffered saline via the lateral tail vein.

Spleen Cell Preparations. Following euthanasia by
cervical dislocation, the spleens of mice were aseptically
transferred to a sterile plastic bag containing Hank's
Balanced Salt Solution. Individual spleens or pooled
spleens from a single experimental group were processed to
single cell suspensions by a 1 minute cycle in a Stomacher
blender. The spleen cell suspensions were washed several
times in either Assay Medium or Stim Medium, as appropriate.
The spleen cells were enumerated by the use of a Coulter
Counter or by trypan blue dye exclusion using a
hemacytometer and microscope.

Sera. Mice were lightly anesthetized with ether and
blood was collected from the retroorbital plexus. Blood
from mice comprising an experimental group was pooled and
allowed to clot. The serum was collected and stored at -
70°C until use.

   *In Vitro Stimulation for the Generation of Secondary
Cytotoxic T Lymphocytes (CTL)*. The pooled spleen cells from
the various experimental groups (responders) were diluted to
5×10^6 cells/ml in Stim Medium. The spleen cells from
syngeneic, naive mice (stimulators) were diluted to 1×10^7
cells per ml and infected for 1 hour in tissue culture
medium containing 2% FBS at 37°C with the appropriate
vaccinia virus at a m.o.i. of 25 pfu per cell. Following
infection, the stimulator cells were washed several times in
Stim Medium and diluted to 1×10^6 cells per ml with Stim
Medium. Five mls of stimulator cells and 5 mls of responder
cells were added to a 25 cm^3 tissue culture flask and
incubated upright at 37°C, in 5% CO_2 for 5 days. On the day
of the assay, the spleen cells were washed several times in
Assay Medium and counted on a hemacytometer in trypan blue
with the use of a microscope.

   Target Cell Preparation. For vaccinia specific CTL
activity, tissue culture cells were infected overnight by
incubation at 1×10^7 cells per ml in tissue culture medium
containing 2% FBS at a m.o.i. of 25 pfu per cell for 1 hour
at 37°C. Following incubation, the cells were diluted to
between $1 - 2 \times 10^6$ cells per ml with tissue culture medium
containing 10% FBS and further incubated at 37°C, in 5% CO_2
until use. For HIV specific CTL activity, tissue culture
cells were incubated overnight with 20 μg/ml of peptide HBX2
(American Biotechnologies, Cambridge, MA), SF2 (American
Biotechnologies, Cambridge, MA) or MN (American
Biotechnologies, Cambridge, MA) corresponding to the V3 loop
region of gp120 of HIV-1 isolates III_B, SF2, and MN,
respectively. On the day of the assay, the targets were
washed several times by centrifugation in Assay Medium.
After the final wash, the cells were resuspended in
approximately 100 μci of $Na_2{}^{51}CrO_4$ (^{51}Cr). Following
incubation at 37°C for 1 hr, the cells were washed at least
3 times in Assay Medium by centrifugation, counted on a
hemacytometer, and diluted to 1×10^5/ml in Assay Medium.

   Cytotoxicity Assays. For primary CTL assays, freshly
prepared spleen cells were diluted with Assay Medium to
1×10^7 cells per ml. For secondary CTL assays (following

either *in vivo* inoculation or *in vitro* stimulation), the
spleen cells were diluted to 2×10^6/ml in Assay Medium. One
tenth ml of spleen cell suspension was added to ^{51}Cr
labelled target cells in the wells of a 96 well, round-
bottom microtiter plate (EXP). In most cases, the spleen
cells being assayed for primary CTL activity were further 2-
fold diluted in the wells of the microtiter plate prior to
the addition of the target cells. As a measure of
spontaneous release of ^{51}Cr (SR), target cells were
incubated in only Assay Medium. To determine the maximum
release of ^{51}Cr (MAX), target cells were deliberately lysed
at the beginning of the assay by adding 0.1 ml of 10% sodium
dodecyl sulfate to the appropriate wells. To initiate the
assay, the microtiter plates were centrifuged at 200 x g for
2 min and incubated for 4 or 5 hrs at 37°C, in 5% CO_2.
Following incubation, the culture supernatants of each well
were collected using the Skatron Supernatant Collection
System. Released ^{51}Cr was determined by a Beckman 5500B
gamma counter. The percent specific cytotoxicity was
determined from the counts by the following formula:

$$\% \text{ CYTOTOXICITY} = (EXP-SR)/(MAX-SR) \times 100$$

Depletion of T Helper Cells and Cytotoxic T Lymphocytes
Using Monoclonal Anti-CD4 and Monoclonal Anti-CD8. Spleen
cell suspensions were diluted to a density of 10^7/ml in
cytotoxicity medium (RPMI 1640 containing 0.2% BSA and 5 mM
HEPES) containing a 1:5 dilution of anti-CD4 (monoclonal
antibody 172.4) or a 1:200 dilution of anti-CD8 (monoclonal
antibody anti-Lyt 2.2) and a 1:16 dilution of Cedar Lane
Low-Tox rabbit complement. Appropriate controls for the
single components (complement, anti-CD4, anti-CD8) were
included.

Anti-HIV-1 gp160 ELISA. The wells of ELISA plates
(Immulon II) were coated overnight at 4°C with 100 ng of
purified HIV-1 gp160 (provided by Dr. D. Bolognesi, Duke
University, Durham NC) in carbonate buffer, pH 9.6. The
plates were then washed with phosphate-buffered saline
containing 0.05% Tween 20 (PBST). The plates were then
blocked for 2 hrs at 37°C with PBST containing 1% bovine
serum albumin (BSA). After washing with PBST, sera were

initially diluted 1:20 with PBST containing 0.1% BSA
(dilution buffer). The sera were further 2-fold serially
diluted in the wells of the ELISA plate. The plates were
incubated at 37°C for 2 hrs and washed with PBST.
Horseradish peroxidase conjugated rabbit anti-mouse
immunoglobulins (DAKO) was diluted 1:2000 in dilution buffer
and added to the wells of the ELISA plate and incubated at
37°C for 1 hour. After washing with PBST, OPD (o-
phenylenediamine dihydrochloride) in substrate buffer was
added and the color was allowed to develop at ambient
temperature for about 20 minutes. The reaction was
extinguished by the addition of 2.5 M H_2SO_4. The absorbance
at 490 nm was determined on a Bio-Tek EL-309 ELISA reader.
The serum endpoint was defined as the reciprocal of the
dilution giving an absorbance value of 1.0.

      Lymphocyte Proliferation Assays. Single cell
suspensions of the spleen cells of individual mice were
diluted to 2×10^6/ml in Assay Medium and 0.1 ml was added to
the wells of 96 well, flat-bottom microtiter plates
containing Assay Medium alone, 1, 5, or 10 μg of HIV-1
peptide T1, 1, 5, or 10 μg of HIV-1 peptide T2, and 1 or 10
μg of purified HIV-1 gp160 (Immuno). The cells were
incubated for 5 days at 37°C, in 5% CO_2. To each well was
added 1.0 μCi of [^{3}H]-thymidine for the final 6 hrs of
incubation and then harvested onto Beckman Ready Filters
using a Cambridge PHD cell harvester. The filter disks were
dry-counted in a liquid scintillation counter.

            STIMULATION INDEX = $CPMs_{EXP}/CPMs_{MEDIUM}$

Results: A Recombinant Vaccinia Virus Expressing HIV gp120
Elicits Primary HIV-specific Cytotoxic T Lymphocyte
Activity. Following iv administration with 5×10^7 PFUs of
vaccinia virus recombinants vP878, vP911, or vP921, or, as a
control, with NYVAC, the vector, splenic CTL activity of
BALB/c mice was assessed against syngeneic P815 cells which
had been incubated overnight with peptide HBX2 (Table 20).
Modest, but significant (P<0.05) primary CTL activity was
generated in the spleens of mice administered vP921,
expressing HIV gp120. No other recombinant vaccinia virus
nor the NYVAC parent vector was able to elicit primary HIV-

specific CTL activity. This was not due to inadequate
infection as each group of mice administered a vaccinia
virus responded with primary vaccinia-specific CTL activity.
Control, unimmunized mice responded to neither target.

   Recombinant Poxviruses Expressing HIV env Peptides
Generate HIV-Specific Memory Cytotoxic T Lymphocytes. At
least one month following a single inoculation with one of
the recombinant vaccinia viruses, mouse spleen cells were
stimulated in vitro with syngeneic, naive spleen cells
previously infected with NYVAC or with each of the HIV
recombinant vaccinia viruses (Table 21). Strong HIV-
specific CTL activity was detected only in the spleen cell
cultures of mice immunized with vP878, vP911, and vP921
which were restimulated in vitro by cells infected with one
of the same vaccinia virus HIV recombinants (vP878, vP911,
or vP921). The vaccinia virus recombinants expressing HIV
gp120 or gp160 were better able to generate memory CTLs than
the vaccinia virus recombinant expressing only the V3 loop
fused to the 88 epitope. HIV-specific memory CTL activity
could not be elicited from unimmunized control or NYVAC
immunized spleen cells. The absence of HIV-specific CTL
activity from vector immunized mice could not be attributed
to poor immunization since vaccinia-specific memory CTL
activity was apparent after in vitro stimulation with spleen
cells infected with any of the vaccinia viruses used.

   In a similar study, the ability of a canarypox
recombinant expressing the V3 loop region fused to the 88
epitope (vCP95) to generate HIV-specific memory CTLs was
examined (Table 22). Three weeks following a single
inoculation of 10^8 PFUs of vCP95 or the ALVAC vector, CPpp,
HIV-specific memory CTL responses were compared to that
elicited by the recombinant vaccinia virus analog, vP878.
Vaccinia and canarypox CTL responses were included as
controls for proper immunization. Only spleen cells from
vP878 and vCP95 immunized mice produced HIV-specific memory
CTL activity which could be stimulated by vP878. The
inability of vCP95 to stimulate existing memory CTLs to
functional cytolytic CTLs may have been related to the in
vitro conditions employed which were maximized based upon

the use of vaccinia virus recombinants. Nonetheless, vCP95
was fully capable of generating significant HIV-specific
memory CTLs in the spleens of immunized mice.

    <u>Characterization of the *In Vitro* Stimulated Cytotoxic
Cells</u>. It is conceivable that the cells mediating
cytotoxicity against the HIV peptide-pulsed target cells
represent a population of nonspecific effector cells
unrelated to CTLs, such as natural killer cells. To test
this, the spleen cells of mice immunized with vP921 and
restimulated *in vitro* with vP921 infected spleen cells were
depleted of T-lymphocytes bearing surface antigens
characteristic of T helper lymphocytes (CD4) or of cytotoxic
T lymphocytes (CD8) and assayed against V3 loop peptide
pulsed target cells (Table 23). As before, only vP921
immunized mice generated memory HIV-specific CTL activity
which could be stimulated *in vitro* with vP921 infected
syngeneic spleen cells. Although the complement preparation
(C') and the monoclonal anti-CD4 and anti-CD8 produced some
toxic effects, only the cultures depleted of CD8-bearing
cells (anti-CD8 + C') were also depleted of HIV-specific
cytotoxic effector cells. Thus, the cells mediating
cytolytic activity against the HIV peptide-pulsed target
cells possessed CD8 antigens on their cell membranes, a
characteristic of MHC class I restricted CTLs.

    <u>Specificity of CTL Antigen Receptor Recognition of the
V3 Loop Region of HIV gp120</u>. T lymphocyte antigen receptors
are exquisitely sensitive to small alterations in the
primary amino acid sequence of the epitope fragment. The V3
loop region of HIV gp120 is hypervariable and differs
immunologically among HIV isolates. The hypervariability
resides in substitutions and additions of only a few amino
acids. To examine the specificity of cytotoxic cells
generated by HIV vaccinia virus recombinants, susceptibility
to CTL activity was compared among P815 target cells pulsed
with peptides corresponding the V3 loop region of HIV
isolates III$_B$, SF2, and MN. Only immunization with vP911
and vP921 induced HIV specific primary CTL activity (Table
24). Furthermore, HIV specific CTL activity was confined
only to P815 target cells pulsed with peptide corresponding

to the V3 loop of HIV isolate III$_B$. Similar results were
obtained with in vitro stimulated, HIV specific secondary
CTL activity induced by immunization with the vaccinia virus
recombinants vP878, vP911, and vP921 (Table 25). Thus, HIV
specific CTLs elicited by recombinant vaccinia viruses
expressing various portions of the env gene of HIV isolate
III$_B$ recognize only target epitopes derived from the same
antigenic isolate.

   Lymphocyte Proliferation Responses to HIV Epitopes
Following Immunization with Vaccinia Virus HIV Recombinants.
Lymphocyte proliferation to antigens is an in vitro
correlate of cell-mediated immunity. Presentation of the
appropriate antigen induces cellular proliferation in the
immune population of cells expressing receptors for the
antigen. The initiation and continuation of proliferation
requires the involvement of T helper lymphocytes via soluble
mediators. To evaluate cell-mediated immunity to HIV
antigens in mice immunized with recombinant vaccinia viruses
expressing HIV antigens, spleen cells from mice immunized 27
days earlier were incubated for 5 days with peptides
correlating to T helper lymphocyte epitopes designated T_1
and T_2, as well as with purified HIV gp160 (Table 26). No
proliferative responses to the T helper cell epitopes T_1 and
T_2 were observed in any of the spleen cell cultures.
However, the spleen cells of mice previously immunized with
vP921 vigorously responded to HIV gp160 as determined by the
incorporation of [^{3}H]-thymidine. A stimulation index (SI)
of greater than 2.0 is considered indicative of immunity.
Thus, inoculation of mice with vP921 elicited cell-mediated
immunity to HIV gp160.

   Antibody Responses of Mice Inoculated with Vaccinia
Virus HIV Recombinants. To evaluate humoral responses to
HIV, mice were immunized at day 0 with one of the vaccinia
virus HIV recombinants and received a secondary immunization
at week 5. The mice were bled at various intervals through
9 weeks after the initial immunization. Pooled sera from
each treatment group were assayed for antibodies to HIV by
ELISA employing purified gp160 as antigen (Table 27).
Primary antibody responses were generally modest, but

detectable with the highest levels induced by vP911.
Following the secondary immunization, the antibody titers of
mice immunized with vP911 and vP921 increased and peaked at
week 7 with titers of over 4,600 and 3,200, respectively,
before declining slightly by week 9. Thus, two vaccinia
virus HIV recombinants, vP911 and vP921, were capable of
inducing a significant antibody response.

Table 20. Primary CTL activity of spleen cells from mice
         immunized with vaccinia virus recombinants against
         vaccinia virus infected targets and targets pulsed
         with peptide corresponding to the V3 loop region
         of HIV-1 gp120.

| | PERCENT CYTOTOXICITY | | |
| | TARGET | | |
IMMUNIZATION	P815	VAC	HIV V3
NONE	-3.5 ± 2.0	-0.6 1.5	-4.8 1.6
NYVAC	-4.4 ± 1.9	9.5 * 3.2	-5.9 1.7
vP878	-4.9 ± 1.8	7.1 * 2.2	-4.0 1.2
vP911	-4.0 ± 2.5	4.6 * 2.0	1.4 5.1
vP921	-3.4 ± 0.9	10.7 * 1.5	15.5 * 2.8

E:T = 100:1
* P<0.05 vs appropriate controls, Student's t-test

Table 21. Secondary CTL activity of spleen cells
          following *in vitro* stimulation with
          vaccinia virus recombinants.

| IMMUNIZATION | | PERCENT CYTOTOXICITY | | |
| | | TARGET | | |
in vivo	*in vitro*	P815	VAC	HIV V3
NONE	NONE	-0.1	1.9	0.5
	NYVAC	3.7	8.9	3.8
	vP878	4.6	9.0	5.5
	vP911	-1.7	2.9	4.8
	vP921	2.9	2.9	1.5
NYVAC	NONE	0.0	4.4	1.1
	NYVAC	3.5	47.8 *	9.2
	vP878	6.3	44.1 *	14.4
	vP911	7.9	48.6 *	10.6
	vP921	6.8	50.8 *	7.9
vP878	NONE	0.1	1.7	1.3
	NYVAC	10.2	58.5 *	13.0
	vP878	11.6	57.9 *	59.9 *
	vP911	7.8	56.2 *	40.8 *
	vP921	4.9	42.0 *	14.8
vP911	NONE	0.3	2.9	4.0
	NYVAC	6.2	50.7 *	8.5
	vP878	5.9	50.9 *	77.4 *
	vP911	5.0	54.2 *	82.6 *
	vP921	10.9	55.0 *	87.8 *
vP921	NONE	2.9	5.0	9.4
	NYVAC	8.3	54.4 *	22.7
	vP878	10.4	56.2 *	85.6 *
	vP911	8.7	58.2 *	86.5 *
	vP921	7.8	55.2 *	81.0 *

BALB/cJ spleen cells from mice immunized approximately 1 month
earlier with the indicated vaccinia virus recombinants were
incubated with infected syngeneic spleen cells for 5 days and
assayed for cytotoxicity at an effector to target cell ratio of
20:1.
o *P*<0.05 compared to controls, Student's *t*-test.

Table 22. Anamnestic CTL responses of the spleen
cells of mice administered a single
inoculation of recombinant vaccinia or
canarypox virus expressing the V3 loop of
HIV gp120.

		PERCENT CYTOTOXICITY			
IMMUNIZATION			TARGET		
PRIMARY *in vivo*	BOOSTER *in vitro*	P815	Vac	CP	HIV V3
NONE	NONE	0.4	-2.5	-2.3	-1.5
	vP804	0.5	8.8	0.7	0.8
	vP878	1.8	6.1	0.4	1.6
	CP	5.8	4.2	4.9	0.4
	vCP95	4.4	2.6	6.1	0.1
	SB135	-0.2	-0.7	-0.4	0.5
vP804	NONE	0.7	1.7	0.1	1.3
	vP804	5.5	43.5 *	5.8	3.5
	vP878	3.6	42.5 *	1.6	-0.3
	CP	8.5	7.0	5.6	3.9
	vCP95	5.8	5.3	4.4	4.0
	SB135	1.2	-0.9	-0.5	-0.2
vP878	NONE	0.2	-2.9	-0.8	-0.2
	vP804	5.3	56.4 *	7.5	4.1
	vP878	6.7	60.2 *	7.7	41.7 *
	CP	8.7	13.4	9.4	4.7
	vCP95	7.1	10.5	8.7	19.0
	SB135	1.9	-0.7	-0.2	-1.4
CP	NONE	4.6	-0.6	2.3	-0.0
	vP804	11.0	17.7 *	5.7	6.1
	vP878	7.1	14.6 *	12.3	5.5
	CP	7.4	5.9	19.3 *	3.1
	vCP95	6.8	5.4	20.4 *	2.8
	SB135	1.4	-0.4	0.8	-1.4
vCP95	NONE	-0.8	-2.2	-1.3	0.3
	vP804	9.4	26.4 *	9.3	6.6
	vP878	10.4	22.5 *	16.9	32.1 *
	CP	8.8	7.2	20.0 *	3.2
	vCP95	5.1	4.2	19.6 *	7.8
	SB135	1.9	-1.5	-0.3	-1.2

Twenty-three days after immunization, the spleen cells were
stimulated *in vitro* for 5 days with virus infected or peptide-
pulsed syngeneic spleen cells and then assayed for specific
cytotoxicity against virus infected or peptide-pulsed P815
target cells at an effector to target cell ratio of 20:1.
* $P<0.05$ compared to appropriate controls, Student's t-test.

Table 23. Depletion of cytotoxic activity with monoclonal
          antibodies to CD8 plus complement.

| IMMUNIZATION | | | PERCENT CYTOTOXICITY | | |
| | | | TARGETS | | |
in vivo	in vitro	TREATMENT	P815	VAC	HIV V3
NONE	NONE	NONE	1.1	1.5	−0.3
NONE	NYVAC	NONE	−7.4	0.4	−0.4
NONE	vP921	NONE	−0.2	1.1	−0.7
NYVAC	NONE	NONE	−3.1	−0.3	−1.4
NYVAC	NYVAC	NONE	−2.6	40.5	−0.3
NYVAC	vP921	NONE	3.3	31.4	−2.9
vP921	NONE	NONE	3.0	−1.3	−0.1
vP921	NYVAC	NONE	−4.9	25.9	12.2
vP921	vP921	NONE	−0.2	21.3	30.5
vP921	vP921	C'	4.6	20.1	22.9
vP921	vP921	anti-CD4	4.2	22.6	23.2
vP921	vP921	anti-CD8	−5.0	22.5	26.9
vP921	vP921	anti-CD4+C'	10.0	26.6	30.1
vP921	vP921	anti-CD8+C'	9.2	7.1	2.3

Table 24. Specificity of primary CTL activity for the V3 loop
         of HIV-1 isolate III$_B$ following a single inoculation
         with HIV recombinant vaccinia viruses.

IMMUNIZATION		PERCENT CYTOTOXICITY			
			TARGET		
				V3 PEPTIDE	
		P815	IIIB	SF2	MN
NONE		-2.7	-1.9	-0.9	-1.2
	±	0.5	0.5	0.5	0.5
NYVAC		-1.6	-0.3	-0.6	-0.3
	±	0.5	0.8	0.7	0.2
vP878		-2.8	0.5	-0.5	-1.2
	±	0.8	1.0	0.6	0.5
vP911		-2.6	7.5 *	-0.5	-1.1
	±	0.2	3.2	0.5	0.4
vP921		-2.5	12.5 *	-0.1	-1.2
	±	0.7	3.6	0.5	0.5

Mice were administered a single iv inoculation with
the indicated vaccinia virus recombinant and assayed
for CTL activity 7 days later against P815 targets
and P815 targets pulsed with one of three peptides
corresponding to the V3 loop region of HIV-1 isolates
III$_B$, SF2, and MN. Although assayed at effector to
target cell ratios of 100:1, 50:1, and 25:1, only the
100:1 data are shown.
* $P < 0.05$ vs appropriate controls, Student's t-test

Table 25. Specificity of secondary CTL activity for
the V3 loop of HIV-1 isolate III$_B$
following a single inoculation with HIV
recombinant vaccinia viruses.

IMMUNIZATION		PERCENT CYTOTOXICITY			
			TARGET		
				V3 PEPTIDE	
in vivo	*in vitro*	P815	IIIB	SF2	MN
NONE	NONE	1.0	1.1	0.5	-0.0
	NYVAC	0.4	0.5	-0.6	-0.3
	vP878	0.2	0.2	-0.5	-1.0
	vP911	-1.5	0.3	-0.5	0.2
	vP921	-0.6	1.4	0.1	-0.5
NYVAC	NONE	-2.2	0.2	0.5	-1.0
	NYVAC	3.2	2.2	3.9	2.5
	vP878	4.4	5.9	5.0	6.1
	vP911	5.8	11.1	5.0	5.3
	vP921	5.0	6.5	2.9	2.9
vP878	NONE	0.1	-0.2	-0.9	-1.0
	NYVAC	3.0	4.8	4.4	4.5
	vP878	7.9	20.2	7.8	8.6
	vP911	4.8	7.8	4.5	4.7
	vP921	2.7	6.9	2.8	3.0
vP911	NONE	0.9	1.8	1.4	0.5
	NYVAC	8.8	8.3	8.1	6.6
	vP878	6.6	57.2	6.8	8.2
	vP911	4.6	63.7	2.9	4.2
	vP921	7.2	63.6	4.1	4.9
vP921	NONE	0.5	0.8	1.2	0.6
	NYVAC	4.4	7.9	7.5	6.0
	vP878	8.1	59.0	7.1	7.5
	vP911	6.4	71.4	7.9	6.6
	vP921	9.3	63.4	9.0	8.1

Table 26. Lymphocyte proliferative responses to HIV gp160 epitopes 27 days after a single immunization with HIV recombinant Vaccinia viruses.

IMMUNIZATION		RPMI	gp160 (ug)		HIV ANTIGEN T1 (ug)			T2 (ug)		
			1	10	1	5	10	1	5	10
NONE	MEAN	5,185	6,397	7,808	7,682	8,614	11,541	6,141	8,835	6,774
	±SD	1,020	2,174	2,596	2,033	2,033	2,036	2,103	1,803	2,806
	SI	1.0	1.2	1.5	1.0	1.1	1.5	1.0	1.4	1.1
MVAC	MEAN	10,327	13,589	15,969	11,360	12,654	15,369	10,339	9,854	8,868
	±SD	1,543	3,323	4,583	1,352	2,272	1,821	762	1,731	502
	SI	1.0	1.3	1.5	1.0	1.1	1.4	1.0	1.0	0.9
vP878	MEAN	10,126	13,150	18,329	11,114	11,956	13,754	10,415	11,442	9,147
	±SD	1,269	1,105	4,245	1,217	1,106	1,568	335	1,288	1,035
	SI	1.0	1.3	1.8	1.0	1.1	1.2	1.0	1.1	0.9
vP911	MEAN	12,155	15,564	26,083	12,417	15,380	17,007	10,681	11,412	10,702
	±SD	1,307	9,707	16,327	873	1,847	6,266	2,428	3,201	1,468
	SI	1.0	1.3	2.1	1.0	1.2	1.4	1.0	1.1	1.0
vP921	MEAN	9,701	49,255*	61,035*	10,550	15,367	15,816	8,818	9,232	8,803
	±SD	2,601	23,673	25,866	3,447	3,481	7,176	954	2,265	2,860
	SI	1.0	5.1	6.3	1.0	1.5	1.5	1.0	1.0	1.0

COUNTS PER MINUTE

SI - stimulation index.
□ $P < 0.05$ compared to unstimulated control cultures
Student's t-test

Table 27. HIV gp160 ELISA titers of mice immunized with HIV recombinant vaccinia viruses.

IMMUNIZATION	WEEKS AFTER IMMUNIZATION						
	0	1	2	4	7	9
CONTROLS	22	32	32	38	36	33
NYVAC	38	36	37	28	50	45
vP878	20	43	27	46	65	63
vP911	0	0	90	453	4,614	3,263
vP921	0	26	25	77	2,614	1,689

SUBSTITUTE SHEET

<u>Example 19</u> - EXPRESSION OF THE HIV-1 (ARV-2 OR SF-2
                STRAIN) env GENE IN ALVAC, TROVAC AND NYVAC
                VECTORS

     <u>Plasmid Constructions</u>. The lambda clone containing the
entire HIV-1 (ARV-2 or SF-2 strain) genome was provided by
J. Levy and was described previously (Sanchez-Pescador et
al., 1985). The <u>env</u> sequences were subcloned into pUC13,
creating plasmid pMP7MX373, which contains the sequences
from -1 relative to the initiation codon (ATG) of the <u>env</u>
gene product to 715 bp downstream of the termination codon
(TAA) of the <u>env</u> gene. These <u>env</u> sequences were excised
from pMP7MX373 by digestion with <u>Eco</u>RI and <u>Hind</u>III and
inserted into the plasmid vector, pIBI25 (International
Biotechnologies, Inc., New Haven, CT), generating plasmid
pIBI25<u>env</u>.
Recombinant plasmid pIBI25<u>env</u> was used to transform
competent <i>E. coli</i> CJ236 (dut- ung-) cells. Single-stranded
DNA was isolated from phage derived by infection of the
transformed <i>E. coli</i> CJ236 cells with the helper phage,
MG408. This single-stranded template was used <i>in vitro</i>
mutagenesis reactions (Kunkel et al., 1985) with
oligonucleotide MUENVT12 (SEQ ID NO:157) (5'-AGAGGGG
AATTCTTCTACTGCAATACA-3'). Mutagenesis with this
oligonucleotide generates a T to C transition and disrupts
the T5CT motif at nucleotide positions 6929-6935 of the ARV-
2 genome (Sanchez-Pescador et al, 1985). This mutation does
not alter the amino acid sequence of the <u>env</u> gene and
creates an <u>Eco</u>RI site, which was used to screen for
mutagenized plasmid clones. Sequence of confirmation was
done by the dideoxynucleotide chain termination method
(Sanger et al., 1977). The resultant mutagenized plasmid
was designated as pIBI25mut<u>env</u>11.

     A 1.45 kb <u>Bgl</u>II fragment was derived from
pIBI25mut<u>env</u>11. This fragment contained the mutated <u>env</u>
sequences. It was used to substitute for the corresponding
unmutated fragment in pIBI<u>env</u>. The resultant plasmid was
designated as pIBI25mut<u>env</u>8. Further modifications were
made to pIBImut<u>env</u>8. <i>In vitro</i> mutageneses were performed to
remove the sequence coding for the rex protein and the LTR
sequence (LTR region) from the 3'-end of the gene and to

delete the putative immuno-suppressive (IS) region amino
acids 583 through 599) (SEQ ID NO:158) Leu-Gln-Ala-Arg-Val-
Leu-Ala-Val-Glu-Arg-Tyr-Leu-Arg-Asp-Gln-Gln-Leu) (Klasse et
al., 1988). These reactions were done with the single-
stranded template derived from pIBImut_env8 with
oligonucleotides LTR2 (SEQ ID NO:159) (5'-TTGGAAAGGCTTTTG-
GCATGCCACGCGTC-3') and MUENSVISR (SEQ ID NO:160) (5'-ACAG
TCTGGGGCATCAAGCAGCTAGGGATTTGGGGTTGCTCT-3'). Mutagenized
clones were identified by hybridization and restriction
analysis. A clone mutagenized such that it was deleted both
of the IS and the LTR region and another deleted of the LTR
was confirmed by nucleotide sequence analysis and designated
pIBI25mut3_env40 and pIBI25mut2_env22, respectively.

     A 3.4 kb _SmaI/HindIII fragment containing the entire
_env gene was derived from pIBI25mut3_env40 and from
pIBImut2_env22 and inserted into pCPCV1, digested with
_SmaI/HindIII. The plasmid pCPCV1 is an insertion plasmid
which enables the generation of CP recombinants. The
foreign genes were directed to the C3 insertion locus.
Plasmids pCPCV1 and pFPCV2 have been described previously in
PCT International Publication No. WO 89/03429 published
April 20, 1989, incorporated herein by reference.

     Oligonucleotide PROVECNS (SEQ ID NO:161) (5'-CCGTTA
AGTTTGTATCGTAATGAAAGTGAAGGGGACCAGG-3') was used for _in vitro
mutagenesis reactions via the method of Mandecki (1986) to
made a precise ATG:ATG construction with the VVH6 promoter
and the _env sequences. Potential mutants were screened for
the loss of the _SmaI site. Plasmid clones devoid of a _SmaI
site were identified and confirmed by nucleotide sequence
analysis. Properly mutagenized plasmid clones were
identified and designated as pCP_envIS+ or pCV_envIS- and
pFP_envIS+ or pFP_envIS-.

     The HIV-1 _env genes were excised from pCP_envIS- by
digestion with _NruI and _HindIII. The two _env fragments of
2.5 kb (_envIS+) and 2.4 kb (_envIS-), respectively, were
isolated and blunt-ended by reaction with the Klenow
fragment of the _E. coli DNA polymerase in the presence of 2
mMdNTPs. These fragments were ligated with the 3.5 kb
fragment derived by digestion of pSIV_envVV with _NruI and

PstI with a subsequent blunting step with the Klenow
fragment of the _E. coli_ DNA polymerase in the presence of
2mM dNTPs. The plasmid pSIVenvVV contains the SIV _env_ gene
expression cassette regulated by the vaccinia virus H6
promoter in the ATI insertion locus. Digestion of pSIV _env_
VV with NruI and PstI excises the entire SIV _env_ coding
sequences and the 3'-most 20 bp of the promoter element.
Ligation to the _env_ IS- and _env_ IS+ fragments restores the
20 bp of the H6 promoter and inserts the HIV-1 _env_ gene into
the ATI insertion plasmid. The resultant plasmids were
designated as pAR5VV+ and pAR6VV- for _env_ IS+ and _env_ IS-,
respectively.

   In Vitro Recombination and Purification of
Recombinants. Recombination was performed introducing
plasmid DNA into infected cells by calcium phosphate
precipitation both for CP (ALVAC) and for FP (TROVAC)
recombinants, as previously described (Piccini et al.,
1987). Plasmids pCPenvIS+ and pCPenvIS- (C5 locus, FIG. 16)
were used to make recombinants vCP61 and vCP60 respectively.
Plasmids pFPenvIS+ and pFPenvIS- (F7 locus, FIG. 22) were
used to make recombinants vFP63 and vFP62, respectively.
The plasmids pAR5VV+ and pAR6VV were used in _in vitro_
recombination experiments with vP866 (NYVAC) as rescue to
yield vP939 and vP940, respectively. Recombinant plaques
were selected by autoradiography after hybridization with a
^{32}P-labeled _env_ specific probe and passaged serially three
times to assure purity, as previously described (Piccini et
al., 1987).

   Radioimmunoprecipitation Analysis. Cell monolayers
were infected at 10 pfu/cell in modified Eagle's methionine-
free medium (MEM met-). At 2 hours post-infection, 20
uCi/ml of [^{35}S]-methionine were added in MEM (-met)
containing 2% dialysed fetal bovine serum (Flow). Cells
were harvested at 15 hrs post-infection by resuspending them
in lysis buffer (150 mM NaCl, 1mM EDTA pH 8, 10 mM Tris-HCl
pH 7.4, 0.2 mg/ml PMSF, 1% NP40, 0.01% Na Azide) and 50 μl
aprotinin, scraped into eppendorf tubes and the lysate was
clarified by spinning 20 minutes at 4°C. One third of the
supernatant of a 60 mm diameter Petri dish was incubated

with 1 µl normal human serum and 100 µl of protein A-
Sepharose CL-4B (SPA) (Pharmacia) for 2 hours at room
temperature. After spinning for 1 minute, the supernatant
was incubated for 1 h 30 minutes at 4°C with 2 µl
preadsorbed human serum from HIV seropositive individuals
(heat-inactivated) and 100 µl SPA.

The pellet was washed four times with lysis buffer and
two times with lithium chloride/urea buffer (0.2 M LiCl, 2 M
urea, 10 mM Tris-HCl pH 8) and the precipitated proteins
were dissolved in 60 µl Laemmli buffer (Laemmli, 1970).
After heating for 5 minutes at 100°C and spinning for 1
minute to remove Sepharose, proteins in the supernatant were
resolved on an SDS 10% polyacrylamide gel and fluorographed.

Expression of the HIV-1 env Gene. Six different
recombinant viruses were prepared where the HIV env gene of
the ARV-2 or SF-2 strain was inserted downstream from a
vaccinia early-late promoter, H6. For simplicity, the two
ALVAC-based recombinant viruses, vCP61 and vCP60, will be
referred to as CPIS+ and CPIS-, the two TROVAC-based
recombinants, vFP63 and vFP62, as FPIS+ and FPIS-, and the
two NYVAC-based recombinants vP939 and vp940 as VV- and VV+,
respectively.

All the constructs were precise, in that, the ATG
initiation codon of the HIV-1 env gene was superimposed on
the ATG of the vaccinia H6 promoter. Moreover, all
extraneous genetic information 3' to the termination codon
was eliminated. CPIS-, FPIS-, and VV- were all obtained by
deletion of a 51 bp region, corresponding to amino acids
583-599, located near the 5' portion of the gp41 gene
product. This region shares homology with putative
immunosuppressive regions (Klasse et al., 1988, Ruegg et
al., 1989b) occurring in the transmembrane polypeptide of
other retrovirus glycoproteins (Cianciolo et al., 1985;
Ruegg et al., 1989a,b).

Expression analyses with all six recombinant viruses
were performed in CEF, Vero, and MRC-5 cell monolayers.
Immuno-precipitation experiments using sera pooled from HIV
seropositive individuals were performed as described in
Materials and Methods. All six recombinants directed the

synthesis of the HIV-1 gp161 envelope precursor. The
efficiency of processing of gp160 to gp120 and gp41,
however, varied between cell types and was also affected by
deletion of the immunosuppressive region. Recognition of
gp41 by the pooled sera from HIV seropositive individuals
also varied with the virus background and the cell type.

**Example 20   -   EXPRESSION OF THE HIV-2 (ISSY STRAIN) env
                   GENE IN NYVAC**

     Expression of gp160. Oligonucleotides HIV25PA (SEQ ID
NO: 162) (5'-
ATGAGTGGTAAAATTCAGCTGCTTGTTGCCTTTCTGCTAACTAGTGCTTGCTTA-3')
and HIV25PB (SEQ ID NO:163) (5'-
TAAGCAAGCACTAGTTAGCAGAAAGGCAACAAGCAGCTGAATTTTACCACTCAT-3')
were annealed to constitute the initial 54 bp of the HIV-2
SBL/ISY isolate (Franchini et al., 1989) env coding
sequence. This fragment was fused 3' to a 129 bp fragment
derived by PCR with oligonucleotides H65PH (SEQ ID NO:164)
(5'-ATCATCAAGCTTGATTCTTTATTCTATAC-3') and H63PHIV2 (SEQ ID
NO:165) (5'-CAGCTGAATTTTACCACTCATTACGATACAAACTTAACG-3')
using pTP15 (Guo et al., 1989) as template. The fusion of
these two fragments was done by PCR using oligonucleotides
HIV25PC (SEQ ID NO:166) (5'-TAAGCAAGCACTAGTTAG-3') and H65PH
(SEQ ID NO:164). The 174 bp PCR derived fragment was
digested with HindIII and SacI and inserted into pBS-SK
(Stratagene, La Jolla, CA) digested with HindIII and SacI.
The resultant fragment was designated pBSH6HIV2. The insert
was confirmed by nucleotide sequence analysis.

     The 3' portion of the HIV-2 env gene was also derived
by PCR. In this reaction a 270 bp fragment was amplified
with oligonucleotides HIV2B1 (SEQ ID NO:167) (5'-
CCGCCTCTTGACCAGAC-3') and HIV2B2 (SEQ ID NO:168) (5'-
ATCATCTCTAGAATAAAAATTACAGGAGGGCAATTTCTG-3') using pISSY-KPN
(provided by Dr. Genoveffa Franchini, NCI-NIH, Bethesda, MD)
as template. This fragment was digested with BamHI and
XbaI. The 150 bp fragment derived from this digestion
contained a 5' BamHI and a 3' XbaI cohesive end. The
fragment was engineered to contain a T5NT sequence motif
known to be recognized as vaccinia virus early transcription

termination signal (Yuen et al., 1987), following the
termination codon (TAA).

The majority of the HIV-2 env gene was obtained from
pISSY-KPN by digestion with SacI and BamHI. The 2.7 kb
fragment generated by this digestion was coinserted into
pBS-SK digested with SacI and XbaI with the 150 bp
BamHI/XbaI fragment corresponding to the 3' end of the
gene. The resultant plasmid was designated pBSHIV2ENV.

The 174 bp SpeI/HindIII fragment from pBSH6HIV2 and the
2.5 kb SpeI/XbaI fragment from pBSHIV2ENV were ligated into
pBS-SK digested with HindIII and XbaI to yield pBSH6HIV2ENV.
The 2.7 kb HindIII/XbaI insert from pBSH6HIV2ENV was
isolated and blunt-ended with the Klenow fragment of the E.
coli DNA polymerase in the presence of 2mM dNTP. The blunt-
ended fragment was inserted into a SmaI digested pSD5HIVC
insertion vector. The resultant plasmid was designated as
pATIHIV2ENV. This plasmid was used in vitro recombination
experiments with vP866 (NYVAC) as the rescuing virus to
yield vP920.

Immunoprecipitation analysis was performed to determine
whether vP920 expresses authentic HIV-2 gp160. Vero cell
monolayers were either mock infected, infected with the
parental virus vP866, or infected with vP920 at an m.o.i. of
10 PFU/cell. Following an hour adsorption period, the
inoculum was aspirated and the cells were overlayed with 2
mls of modified Eagle's medium (minus methionine) containing
2% fetal bovine serum and [^{35}S]-methionine (20 μCi/ml).
Cells were harvested at 18 hrs post-infection by the
addition of 1 ml 3X buffer A (3% NP-40, 30 mM Tris pH 7.4,
150 mM NaCl, 3mM EDTA, 0.03% Na Azide and 0.6 mg/ml PMSF)
with subsequent scraping of the cell monolayers.

Lysates from the infected cells were analyzed for HIV-2
env gene expression using pooled serum from HIV-2
seropositive individuals (obtained from Dr. Genoveffa
Franchini). The sera was preadsorbed with vP866 infected
Vero cells. The preadsorbed human sera was bound to Protein
A-sepharose in an overnight incubation at 4°C. Following
this incubation period, the material was washed 4X with 1X
buffer A. Lysates precleared with normal human sera and

protein A-sepharose were then incubated overnight at 4°C
with the human sera from seropositive individuals bound to
protein A-sepharose. After the overnight incubation period,
the samples were washed 4X with 1X buffer A and 2X with a
LiCl$_2$/urea buffer. Precipitated proteins were dissociated
from the immune complexes by the addition of 2X Laemmlis
buffer (125mM Tris(pH6.8), 4% SDS, 20% glycerol, 10% 2-
mercaptoethanol) and boiling for 5 min. Proteins were
fractionated on a 10% Dreyfuss gel system (Dreyfuss et al.,
1984), fixed and treated with 1M Na-salicylate for
fluorography.

     Human sera from HIV-2 seropositive individuals
specifically precipitated the HIV-2 gp160 envelope
glycoprotein from vP920 infected cells. Furthermore, the
authenticity of the expressed HIV-2 env gene product was
confirmed, since the gp160 polyprotein is processed to the
mature gp120 and gp41 protein species. No HIV-specific
protein species were precipitated from mock-infected cells
or cells infected with the NYVAC parental virus. Also,
supportive of the proper expression of the HIV-2 env by
vP920 was the observation that the gene product is expressed
on the surface of vP920 infected cells.

     Expression of gp120. The plasmid pBSH6HIV2 containing
the vaccinia virus H6 promoter fused to the 5'-end of the
HIV-2 env gene was digested with SpeI and HindIII to
liberate the 180 bp fragment containing these sequences.
This fragment was ligated into pBS-SK digested with HindIII
and XbaI along with the 1.4 kb SpeI/XbaI fragment of
pBSHIV2120A to yield pSHIV2120B.

     The plasmid pBSHIV2120A was derived by initially
deriving the 3' portion of the gp120 coding sequence by PCR.
The PCR was performed using oligonucleotides HIV2120A (SEQ
ID NO:169) (5'-ATCATCTCTAGAATAAAAATTATCTCTTATGTCTCCCTGG-3')
and HIV2120B (SEQ ID NO:170) (5'-AATTAACTTTACAGCACC-3') with
pISSY-KPN as template. The PCR-derived fragment was
digested with EcoRI and XbaI to yield a 300 bp fragment
which contained a 5'-EcoRI cohesive end and a 3'-XbaI
cohesive end. The fragment was engineered with a
translation termination sequence (TAA) and a T5NT sequence

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motif just 5' to the <u>Xba</u>I site. The 300 bp <u>Xba</u>I/<u>Eco</u>RI PCR
fragment was ligated into pBS-SK digested with <u>Sac</u>I/<u>Xba</u>I
along with a 1.4 kb <u>Sac</u>I/<u>Eco</u>RI fragment derived from pISSY-
KPN to generate pBSHIV2120A.

The plasmid pBSHIV2120B was digested with <u>Hin</u>dIII and
<u>Xba</u>I to generate a 1.8 kb fragment containing the HIV-2
gp120 coding sequence juxtaposed 3' to the vaccinia virus H6
promoter. This fragment was blunted with the Klenow
fragment of the *E. coli* DNA polymerase in the presence of 2
mM dNTPs. The blunt-ended fragment was ligated to <u>Sma</u>I
digested pSDSHIVC to generate pATI HIV 2120. This plasmid
was used in *in vitro* recombination experiments to yield
vP922.

Immunoprecipitation experiments with vP922 infected
cells were performed as described above for the expression
of the entire HIV-2 <u>env</u> gene. No HIV-specific species were
precipitated from mock infected or vP866 infected Vero
cells. A protein species of 120 kDa was, however,
precipitated from lysates derived from cells infected with
vP922. The HIV-2 gp120 expressed by vP920 was found to be
present on the cell surface of vP920 infected Vero cells.

Example 21 - EXPRESSION OF SIV GENES IN NYVAC

Generation of NYVAC/SIV gp140 Recombinant. A plasmid
pSSIIE containing the SIV (Mac$_{142}$) <u>env</u> gene was obtained
from Dr. Genoveffa Franchini (NCI-NIH, Bethesda, MD). This
plasmid was digested with <u>Hin</u>dIII and <u>Pst</u>I to liberate a 2.2
kbp fragment containing from nucleotide 220 of the SIV <u>env</u>
gene to a region 160 bp downstream from the translation
termination codon. It should be noted that an expression
cassette containing this fragment will result in the
expression of a gp140 protein species rather that a gp160
species. This 40% deletion of the transmembrane region
results from a premature termination at nucleotide 7,934 of
the genome (Franchini et al., 1987). Such premature
terminations of the <u>env</u> gene product are noted after
propagation of SIV in culture (Kodama et al., 1989).

The amino portion of the gene was derived by PCR using
pSSIIE as template and oligonucleotides SIVENV1 (SEQ ID
NO:171) (5'-CGATATCCGTTAAGTTTGTATCGTAATGGGATGTCTTGGGAATC-3')

and SIVENV2 (SEQ ID NO:172) (5'-CAAGGCTTTATTGAGGTCTC-3').
The resultant 250 bp fragment contains the 5'-most 230 bp of
the SIV env gene juxtaposed downstream from the 3'-most 20
bp of the vaccinia virus H6 promoter (3'-end of NruI site).
A 170 bp fragment was obtained by digestion of the fragment
with HindIII, which removes 80 bp of SIV env sequences.

     The sequences containing the remainder of the SIV env
gene following the premature termination signal were derived
by PCR from pSS35E (obtained from Dr. Genoveffa Franchini).
This plasmid contains sequences containing the C-terminal
portion of the SIV env gene into the LTR region downstream
from the env gene. The oligonucleotides used to derive the
360 bp fragment were SIVENV3 (SEQ ID NO:173) (5'-
CCTGGCCTTGGCAGATAG-3') and SIVENV4A (SEQ ID NO:174) (5'-
ATCATCGAATTCAAAAATATTACAAAGAGCGTGAGCTCAAGTCCTTGCCTAATCCTCC-
3'). This fragment was digested with PstI and EcoRI to
generate a 260 bp fragment having a 5' PstI cohesive end and
a 3' EcoRI cohesive end.

     The 2.2 kb HindIII/PstI fragment from pSSIIE, the 170
bp NruI/HindIII fragment containing the 5' end of the gene,
and the 260 bp PstI/EcoRI containing the 3' end of the gene
were ligated with a 3.1 kb NruI/EcoRI fragment derived from
pRW838. pRW838 contains the vaccinia virus H6 promoter
linked to the rabies G gene flanked by canarypox virus
sequences which enable the insertion of genes into the C5
locus. Digestion with NruI and EcoRI liberates the rabies G
gene and removes the 3'-most 20 bp of the H6 promoter. The
resultant C5 insertion plasmid containing the SIV env gene
linked to the vaccinia H6 promoter was designated as
pC5SIVENV.

     The plasmid, pC5SIVENV, was digested with HindIII and
EcoRI to liberate a 2.2 kb fragment, containing from
nucleotide 150 of the SIV env gene to the end of the entire
gene. PCR was used to derive the vaccinia H6 promoter/SIV
env linkage from pC5SIVENV with oligonucleotides MPSYN286
(SEQ ID NO:175) (5'-CCCCCCAAGCTTTTTTATTCTATACTT-3') and
SIVENV2 (SEQ ID NO:176) (5'-CAAGGCTTTATTGAGGTCTC-3'). The
320 bp fragment was digested with HindIII to derive a 240 bp
fragment. The 2.2 kb HindIII/EcoRI and the 240 bp HindIII

fragment were coligated into pC3I digested with HindIII and
EcoRI. The resultant plasmid containing the HindIII
fragment in the proper orientation relative to the SIVenv
coding sequence was designated pC3SIVEM. The plasmid pC3I
was derived as follows. The nucleotide sequence analysis of
an 2.5 kb BglII canarypoxvirus genomic fragment revealed the
entire C3 open reading frame and the 5' and 3' noncoding
regions. In order to construct a donor plasmid for
insertion of foreign genes into the C3 locus with the
complete excision of the C3 open reading frame, PCR primers
were used to amplify the 5' and 3' sequences relative to C3.
Primers for the 5' sequences were RG277 (SEQ ID NO:177) (5'-
CAGTTGGTACCACTGGTATTTTATTTCAG-3') and RG278 (SEQ ID NO:178)
(5'-TATCTGAATTCCTGCAGCCCGGGTTTTTATAGCTAATTAGTCAAATGTGAG
TTAATATTAG-3').

    Primers for the 3' sequences were RG279 (SEQ ID NO:179)
(5'-TCGCTGAATTCGATATCAAGCTTATCGATTTTTATGACTAGTTAATCAAATA
AAAAGCATACAAGC-3') and RG280 (SEQ ID NO:180) (5'-
TTATCGAGCTCTGTAACATCAGTATCTAAC-3'). The primers were
designed to include a multiple cloning site flanked by
vaccinia transcriptional and translation termination
signals. Also included at the 5'-end and 3'-end of the left
arm and right arm were appropriate restriction sites (Asp718
and EcoRI for left arm and EcoRI and SacI for right arm)
which enabled the two arms to ligate into Asp718/SacI
digested pBS-SK plasmid vector. The resultant plasmid was
designated as pC3I.

    The plasmid pC3SIVEM was linearized by digestion with
EcoRI. Subsequent partial digestion with HindIII liberated
a 2.7 kb HindIII/EcoRI fragment. This fragment was blunt-
ended by treatment with Klenow fragment of the _E. coli_ DNA
polymerase in the presence of 2mM dNTPs. The fragment was
ligated into pSD550VC digested with SmaI. The resultant
plasmid was designated as pSIVEMVC. This plasmid was used
in _in vitro_ recombination experiments with vP866 as rescue
virus to generate vP873. vP873 contains the SIV env gene in
the I4L locus.

    Generation of a NYVAC/gag/pol and gag Recombinants. A
plasmid, pSIVAGSSIIG, containing the SIV cDNA sequence

encompassing the gag and pol genes was obtained from Dr.
Genoveffa Franchini (NCI-NIH, Bethesda, MD). The gag and
pol genes from this plasmid were juxtaposed 3' to the
vaccinia I3L promoter between vaccinia _tk_ flanking arms.
This was accomplished by cloning the 4,800 bp _Cfo_I/_Taq_I
fragment of pSIVGAGSSIIG, containing the gag and the
oligonucleotides SIVL1 (SEQ ID NO:181) (5'-
TCGAGTGAGATAAAGTGAAAATATATATCATTATATTACAAGTA
CAATTATTTAGGTTTAATCATGGGCG-3') and SIVL2 (SEQ ID NO:182)
(5'-CCCATGATTAAACCTAAATAATTGTACTTTGTAATATAATGCTATATATTTT
CACTTTATCTCAC-3') corresponding to the I3L promoter into the
4,070 bp _Xho_I/_Acc_I fragment of pSD542, a derivative of
pSD460 (FIG. 1). The plasmid generated by this manipulation
was designated pSIVG1.

To eliminate the pol gene, a 215 bp PCR fragment was
derived from pSIVGAGSSIIG using oligonucleotides SIVP5 (SEQ
ID NO:183) (5'-AATCAGAGAGCAGGCT-3') and SIVP6 (SEQ ID
NO:184) (5'-TTGGATCCCTATGCCACCTCTCT-3'). The PCR-derived
fragment was digested with _Bam_HI and _Stu_I and ligated with
the 5,370 bp partial _Bam_HI/_Stu_I fragment of SIVG1. This
resulted in the generation of pSIVG2. pSIVG2 was used in _in
vitro_ recombination experiments with vP873 as rescue virus
to yield vP948.

The plasmid to insert both gag and pol into NYVAC-based
vectors was engineered in the following manner. pSIVG1,
described above, contains extraneous 3'-noncoding sequences
which were eliminated using a 1 kb PCR fragment. This
fragment was generated from plasmid pSIVGAGSSIIG with the
oligonucleotides SIVP5 and SIVP6. This PCR derived fragment
containing the 3' end of the pol gene was digested with
_Bam_HI and _Hpa_I. The 1 kb _Bam_HI/_Hpa_I fragment was ligated to
the 7,400 bp partial _Bam_HI/_Hpa_I fragment of pSIVG1 to yield
pSIVG4.

Sequence analysis of pSIVG4 revealed a single base pair
deletion within the pol gene. To correct this error the
2,300 bp _Bgl_II/_Stu_I fragment from pSIVG1 was inserted into
the 6,100 bp partial _Bgl_II/_Stu_I fragment of pSIVG4 to yield
pSIVG5. The plasmid, pSIVG5, was used in _in vitro_

recombination experiments with vP873 as rescue to generate
vP943.

     Generation of NYVAC/SIV p16 and p28 Recombinants. The
pol gene and the portion of the gag gene encoding p28, p2,
p8, p1, and p6 were eliminated from pSIVG1. This was
accomplished by cloning the oligonucleotides SIVL10 (SEQ ID
NO:185) (5'-AGACCAACAGCACCATCTAGCGGCAGAGGAGGAAATTACTAATTTTT
ATTCTAGAG-3') and SIVL11 (SEQ ID NO:186) (5'-GATCCTCTA
GAATAAAAAATTAGTAATTTCCTCCTCTGCCGCTAGATGGTGCTGTTGGT-3') into
the 4,430 bp AccI/BamHI fragment of pSIVG1 to generate
pSIVG1 to generate pSIVG3. This plasmid contains an
expression cassette for the SIV p17 gene product expressed
by the vaccinia I3L promoter.

     The entomopoxvirus 42 kDa-promoted SIV p28 gene (5' end
only) was inserted downstream from the I3L-promoted p17
gene. This was accomplished by cloning the 360 bp
BspMI/BamHI fragment of pSIVGI, containing the 5' end of the
p28 gene, the oligonucleotides pSIVL14 (SEQ ID NO:187) (5'-
TAGACAAAATTGAAAATATATAATTACAATATAAAATGCCAGTACAACAAATAGGTGGTA
ACTATGTCCACCTGCCATT-3') and SIVL15 (SEQ ID NO:188) (5'-
GCTTAATGGCAGGTGGACATAGTTACCACCTATTTGTTGTACTGGCATTTTATATTGTAA
TTATATATTTTCAATTTTGT-3'), containing the entomopox 42 kDa
promoter into the 4,470 bp partial XbaI/BamHI fragment of
pSIVG3. The resultant plasmid was designated as pSIVG6.

     The 3' portion of the p28 gene was then inserted into
pSIVG6. A 290 bp PCR fragment, containing the 3' end of the
SIV p28 gene, was derived from pSIVGI using oligonucleotides
SIVP12 (SEQ ID NO:189) (5'-TGGATGTACAGACAAC-3') and SIVP13
(SEQ ID NO:190) (5'-AAGGATCCGAATTCTTACATTAATCTAGCCTTC-3').
This fragment was digested with BamHI and ligated to the
4,830 bp BamHI fragment of pSIVG7, was used in in vitro
recombination experiments with vP866 and vP873 as rescue
experiments to generate vP942 and vP952, respectively.

     Expression Analyses. The SIV gp140 env gene product is
a typical glycoprotein associated with the plasma membrane
of infected cells. It is expressed as a polyprotein of 140
kDa that is proteolytically cleaved to an extracellular
species of 112 kDa and a transmembrane region of 28 kDa
(Franchini et al., 1987). Immunofluorescence analysis using

sera from rhesus macaques seropositive for SIV followed by
fluorescein conjugated rabbit anti-monkey IgG demonstrated
expression of the env gene product on the surface of
recombinant infected Vero cells. Surface expression was not
detectable on the surface of mock infected cells or cells
infected with the NYVAC (vP866) parent virus. Furthermore,
cells infected with recombinants containing only gag genes
were not shown to express any SIV components on the surface.
Surface expression in cells infected with vP873, vP943,
vP948 and vP952 all demonstrated surface expression and
significantly, all contain the SIV env gene.

     The authenticity of the expressed SIV gene products
(env and gag) in Vero cells infected with the NYVAC/HIV
recombinants was analyzed by immunoprecipitation. Vero
cells were infected at an m.o.i. of 10 with the individual
recombinant viruses, with the NYVAC parent virus, or were
mock infected. After a 1 hour adsorption period, the
inoculum was removed and infected cells were overlayed with
2 ml methionine-free media containing [^{35}S]-methionine (20
μCi/ml). All samples were harvested at 17 hours post
infection by the addition of 1 ml of 3X Buffer A. Lysates
from the infected cells were analyzed with pooled sera from
SIV seropositive rhesus macaques or a monoclonal antibody
specific for gag p24 gene product (both obtained from Dr.
Genoveffa Franchini, NCI-NIH, Bethesda MD).

     Immunoprecipitation with the SIV seropositive macaque
sera was performed in the following manner. The macaque
sera were incubated with protein A-sepharose at 4°C for 16
hours. After washing with buffer A, the sera bound to
protein A sepharose were added to lysates precleared with
normal monkey sera and protein A sepharose. Following an
overnight incubation at 4°C the precipitates were washed 4 x
with buffer A and 2 x with LiCl/urea buffer. To dissociate
the precipitated protein from the antibody, the samples were
boiled in 80 μl 2 x Laemmli buffer for 5 minutes. The
samples were fractionated on a 12.5% gel using the Dreyfuss
gel system (Dreyfuss et al., 1984). The gel was fixed and
treated with 1 M Na-salycate for fluorography.

All the recombinants containing SIV genes were expressing
the pertinent gene products. The NYVAC recombinants vP873,
vP943, vP948 and vP952 which contain the SIV env gene all
expressed the authentic gp140. However, it is difficult to
assess the processing of the gp140 protein to the 112 kDa
and 28 kDa mature forms. No species with an apparent
molecular weight of 140 kDa was precipitated by macaque
anti-SIV sera from mock infected Vero cells, vP866 infected
Vero cells and Vero cells infected with a NYVAC/SIV
recombinant not containing the SIV env gene. Expression of
the SIV gag encoded gene products by vP942, vp943, vp948,
and vP952 was demonstrated using the pooled sera from
macaques infected with SIV and the monoclonal antibody
specific to the p28 gag component. Expression of the entire
p55 gag protein without the pol region, which contains the
protease function, by NYVAC (vP948) in Vero cells is
evident. These results demonstrate that lack of SIV
protease expression prevents the complete proteolysis of p55
into its mature form. This is demonstrated much more
clearly when a monoclonal antibody specific to p28 was used
to precipitate gag specific gene products from vP948
infected Vero cells. Contrary to this result, expression of
SIV gag with the pol gene (includes protease) in vP943
infected Vero cells enabled the expressed p55 gag precursor
polypeptide to be proteolytically cleaved to its mature
forms.

    Expression of both the p16 and p28 SIV gene products in
vP942 and vP952 infected Vero cells was demonstrated using
the pooled sera from macaques infected with SIV. Using the
monoclonal antibody specific to p28 obviously only
recognized the p28 expressed component.

**Example 22 — CONSTRUCTION OF TROVAC RECOMBINANTS
               EXPRESSING THE HEMAGGLUTININ GLYCOPROTEINS OF
               AVIAN INFLUENZA VIRUSES**

    This Example describes the development of fowlpox virus
recombinants expressing the hemagglutinin genes of three
serotypes of avian influenza virus.

    Cells and Viruses. Plasmids containing cDNA clones of
the H4, H5 and H7 hemagglutinin genes were obtained from Dr.
Robert Webster, St. Jude Children's Research Hospital,

Memphis, Tennessee. The strain of FPV designated FP-1 has
been described previously (Taylor et al., 1988a, b). It is
an attenuated vaccine strain useful in vaccination of day
old chickens. The parental virus strain Duvette was
obtained in France as a fowlpox scab from a chicken. The
virus was attenuated by approximately 50 serial passages in
chicken embryonated eggs followed by 25 passages on chick
embryo fibroblast (CEF) cells. This virus was obtained in
September 1980 by Rhone Merieux, Lyon, France, and a master
viral seed established. The virus was received by
Virogenetics in September 1989, where it was subjected to
four successive plaque purifications. One plaque isolate
was further amplified in primary CEF cells and a stock
virus, designated as TROVAC, was established. The stock
virus used in the *in vitro* recombination test to produce
TROVAC-AIH5 (vFP89) and TROVAC-AIH4 (vFP92) had been further
amplified though 8 passages in primary CEF cells. The stock
virus used to produce TROVAC-AIH7 (vFP100) had been further
amplified through 12 passages in primary CEF cells.

     Construction of Fowlpox Insertion Plasmid at F8 Locus.
Plasmid pRW731.15 contains a 10 kbp PvuII-PvuII fragment
cloned from TROVAC genomic DNA. The nucleotide sequence was
determined on both strands for a 3661 bp PvuII-EcoRV
fragment. This sequence is shown in FIG. 21. The limits of
an open reading frame designated in this laboratory as F8
were determined within this sequence. The open reading
frame is initiated at position 704 and terminates at
position 1888. In order not to interfere with neighboring
open reading frames, the deletion was made from position 781
to position 1928, as described below.

     Plasmid pRW761 is a sub-clone of pRW731.15 containing a
2430 bp EcoRV-EcoRV fragment. The F8 ORF was entirely
contained between an XbaI site and an SspI site in PRW761.
In order to create an insertion plasmid which, on
recombination with TROVAC genomic DNA would eliminate the F8
ORF, the following steps were followed. Plasmid pRW761 was
completely digested with XbaI and partially digested with
SspI. A 3700 bp XbaI-SspI band was isolated and ligated

with the annealed double-stranded oligonucleotides JCA017
(SEQ ID NO:191) and JCA018 (SEQ ID NO:192).
JCA017 (SEQ ID NO:191) 5' CTAGACACTTTATGTTTTTTAATATCCGGTCTT
          AAAAGCTTCCCGGGGATCCTTATACGGGGAATAAT 3'
JCA018 (SEQ ID NO:192) 5' ATTATTCCCCGTATAAGGATCCCCCGGGAA
          GCTTTTAAGACCGGATATTAAAAAACATAAAGTGT 3'

     The plasmid resulting from this ligation was designated
pJCA002. Plasmid pJCA004 contains a non-pertinent gene
linked to the vaccinia virus H6 promoter in plasmid pJCA002.
The sequence of the vaccinia virus H6 promoter has been
previously described (Taylor et al., 1988a, b; Guo et al.
1989; Perkus et al., 1989). Plasmid pJCA004 was digested
with EcoRV and BamHI which deletes the non-pertinent gene
and a portion of the 3' end of the H6 promoter. Annealed
oligonucleotides RW178 (SEQ ID NO:193) and RW179 (SEQ ID
NO:194) were cut with EcoRV and BamHI and inserted between
the EcoRV and BamHI sites of JCA004 to form pRW846.
RW178 (SEQ ID NO:193):   5' TCATTATCGCGATATCCGTGTTAACTAGCTA
                          GCTAATTTTTATTCCCGGGATCCTTATCA 3'
RW179 (SEQ ID NO:194):   5' GTATAAGGATCCCGGGAATAAAAATTAGCT
                          AGCTAGTTAACACGGATATCGCGATAATGA 3'
Plasmid pRW846 therefore contains the H6 promoter 5' of
EcoRV in the de-ORFed F8 locus. The HincII site 3' of the
H6 promoter in pRW846 is followed by translation stop
codons, a transcriptional stop sequence recognized by
vaccinia virus early promoters (Yuen et al., 1987) and a
SmaI site.

     Construction of Fowlpox Insertion Plasmid at F7 Locus.
The original F7 non-de-ORFed insertion plasmid, pRW731.13,
contained a 5.5 kb FP genomic PvuII fragment in the PvuII
site of pUC9. The insertion site was a unique HincII site
within these sequences. The nucleotide sequence shown in
FIG. 22 was determined for a 2356 bp region encompassing the
unique HincII site. Analysis of this sequence revealed that
the unique HincII site (FIG. 22, underlined) was situated
within an ORF encoding a polypeptide of 90 amino acids. The
ORF begins with an ATG at position 1531 and terminates at
position 898 (positions marked by arrows in FIG. 22).

The arms for the de-ORFed insertion plasmid were
derived by PCR using pRW731.13 as template. A 596 bp arm
(designated as HB) corresponding to the region upstream from
the ORF was amplified with oligonucleotides F73PH2 (SEQ ID
NO:195) (5'-GACAATCTAAGTCCTATATTAGAC-3') and F73PB (SEQ ID
NO:196) (5'-GGATTTTTAGGTAGACAC-3'). A 270 bp arm
(designated as EH) corresponding to the region downstream
from the ORF was amplified using oligonucleotides F75PE (SEQ
ID NO:197) (5'-TCATCGTCTTCATCATCG-3') and F73PH1 (SEQ ID
NO:198) (5'-GTCTTAAACTTATTGTAAGGGTATACCTG-3').

Fragment EH was digested with EcoRV to generate a 126
bp fragment. The EcoRV site is at the 3'-end and the 5'-end
was formed, by PCR, to contain the 3' end of a HincII site.
This fragment was inserted into pBS-SK (Stratagene, La
Jolla, CA) digested with HincII to form plasmid pF7D1. The
sequence was confirmed by dideoxynucleotide sequence
analysis. The plasmid pF7D1 was linearized with ApaI,
blunt-ended using T4 DNA polymerase, and ligated to the 596
bp HB fragment. The resultant plasmid was designated as
pF7D2. The entire sequence and orientation were confirmed
by nucleotide sequence analysis.

The plasmid pF7D2 was digested with EcoRV and BglII to
generate a 600 bp fragment. This fragment was inserted into
pBS-SK that was digested with ApaI, blunt-ended with T4 DNA
polymerase, and subsequently digested with BamHI. The
resultant plasmid was designated as pF7D3. This plasmid
contains an HB arm of 404 bp and a EH arm of 126 bp.

The plasmid pF7D3 was linearized with XhoI and blunt-
ended with the Klenow fragment of the *E. coli* DNA polymerase
in the presence of 2mM dNTPs. This linearized plasmid was
ligated with annealed oligonucleotides F7MCSB (SEQ ID
NO:199) (5'-AACGATTAGTTAGTTACTAAAAGCTTGCTGCAGCCCGGGTTT
TTTATTAGTTTAGTTAGTC-3') and F7MCSA (SEQ ID NO:200) (5'-
GACTAACTAACTAATAAAAAACCCGGGCTGCAGCAAGCTTTTTGTAACTAACTAA
TCGTT-3'). This was performed to insert a multiple cloning
region containing the restriction sites for HindIII, PstI
and SmaI between the EH and HB arms. The resultant plasmid
was designated as pF7D0.

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Construction of Insertion Plasmid for the H4

Hemagglutinin at the F8 Locus. A cDNA copy encoding the
avian influenza H4 derived from A/Ty/Min/833/80 was obtained
from Dr. R. Webster in plasmid pTM4H833. The plasmid was
digested with HindIII and NruI and blunt-ended using the
Klenow fragment of DNA polymerase in the presence of dNTPs.
The blunt-ended 2.5 kbp HindIII-NruI fragment containing the
H4 coding region was inserted into the HincII site of pIBI25
(International Biotechnologies, Inc., New Haven, CT). The
resulting plasmid pRW828 was partially cut with BanII, the
linear product isolated and recut with HindIII. Plasmid
pRW828 now with a 100 bp HindIII-BanII deletion was used as
a vector for the synthetic oligonucleotides RW152 (SEQ ID
NO:201) and RW153 (SEQ ID NO:202). These oligonucleotides
represent the 3' portion of the H6 promoter from the EcoRV
site and align the ATG of the promoter with the ATG of the
H4 cDNA.

RW 152 (SEQ ID NO:201):   5' GCACGGAACAAAGCTTATCGCGATATCCGTTA
                             AGTTTGTATCGTAATGCTATCAATCACGATTCTGT
                             TCCTGCTCATAGCAGAGGGCTCATCTCAGAAT 3'

RW 153 (SEQ ID NO:202):   5' ATTCTGAGATGAGCCCTCTGCTATGAGCAGGA
                             ACAGAATCGTGATTGATAGCATTACGATACAAACT
                             TAACGGATATCGCGATAAGCTTTGTTCCGTGC 3'

The oligonucleotides were annealed, cut with BanII and
HindIII and inserted into the HindIII-BanII deleted pRW828
vector described above. The resulting plasmid pRW844 was
cut with EcoRV and DraI and the 1.7 kbp fragment containing
the 3' H6 promoted H4 coding sequence was inserted between
the EcoRV and HincII sites of pRW846 (described previously)
forming plasmid pRW848. Plasmid pRW848 therefore contains
the H4 coding sequence linked to the vaccinia virus H6
promoter in the de-ORFed F8 locus of fowlpox virus.

Construction of Insertion Plasmid for H5 Hemagglutinin

at the F8 Locus. A cDNA clone of avian influenza H5 derived
from A/Turkey/Ireland/1378/83 was received in plasmid pTH29
from Dr. R. Webster. Synthetic oligonucleotides RW10 (SEQ
ID NO:203) through RW13 (SEQ ID NO:206) were designed to
overlap the translation initiation codon of the previously
described vaccinia virus H6 promoter with the ATG of the H5

gene. The sequence continues through the 5' SalI site of
the H5 gene and begins again at the 3' H5 DraI site
containing the H5 stop codon.

RW10 (SEQ ID NO:203):   5' GAAAAATTTAAAGTCGACCTGTTTTGTTGAGT

                          TGTTTGCGTGGTAACCAATGCAAATCTGGTC

                          ACT 3'

RW11 (SEQ ID NO:204):   5' TCTAGCAAGACTGACTATTGCAAAAAGAAGCA

                          CTATTTCCTCCATTACGATACAAACTTAACG

                          GAT 3'

RW12 (SEQ ID NO:205):   5' ATCCGTTAAGTTTGTATCGTAATGGAGGAAA

                          TAGTGCTTCTTTTTGCAATAGTCAGTCTTGCTAGA

                          AGTGACCAGATTTGCATTGGT 3'

RW13 (SEQ ID NO:206):   5' TACCACGCAAACAACTCAACAAAACAGGTCG

                          ACTTTAAATTTTTCTGCA 3'

     The oligonucleotides were annealed at 95°C for three
minutes followed by slow cooling at room temperature. This
results in the following double strand structure with the
indicated ends.

```
EcoRV                                                          PstI
|                RW12                  |              RW13         |

   _____
      RW11                    |              RW10
```

     Cloning of oligonucleotides between the EcoRV and PstI
sites of pRW742B resulted in pRW744. Plasmid pRW742B
contains the vaccinia virus H6 promoter linked to a non-
pertinent gene inserted at the HincII site of pRW731.15
described previously. Digestion with PstI and EcoRV
eliminates the non-pertinent gene and the 3'-end of the H6
promoter. Plasmid pRW744 now contains the 3' portion of the
H6 promoter overlapping the ATG of avian influenza H5. The
plasmid also contains the H5 sequence through the 5' SalI
site and the 3' sequence from the H5 stop codon (containing
a DraI site). Use of the DraI site removes the H5 3' non-
coding end. The oligonucleotides add a transcription
termination signal recognized by early vaccinia virus RNA
polymerase (Yuen et al., 1987). To complete the H6 promoted
H5 construct, the H5 coding region was isolated as a 1.6 kpb
SalI-DraI fragment from pTH29. Plasmid pRW744 was partially
digested with DraI, the linear fragment isolated, recut with

<u>Sal</u>I and the plasmid now with eight bases deleted between
<u>Sal</u>I and <u>Dra</u>I was used as a vector for the 1.6 kpb pTH29
<u>Sal</u>I and <u>Dra</u>I fragment. The resulting plasmid pRW759 was
cut with <u>Eco</u>RV and <u>Dra</u>I. The 1.7 kbp PRW759 <u>Eco</u>RV-<u>Dra</u>I
fragment containing the 3' H6 promoter and the H5 gene was
inserted between the <u>Eco</u>RV and <u>Hinc</u>II sites of pRW846
(previously described). The resulting plasmid pRW849
contains the H6 promoted avian influenza virus H5 gene in
the de-ORFed F8 locus.

    <u>Construction of Insertion Vector for H7 Hemagglutinin</u>
<u>at the F7 Locus</u>. Plasmid pCVH71 containing the H7
hemagglutinin from A/CK/VIC/1/85 was received from Dr. R.
Webster. An <u>Eco</u>RI-<u>Bam</u>HI fragment containing the H7 gene was
blunt-ended with the Klenow fragment of DNA polymerase and
inserted into the <u>Hinc</u>II site of pIBI25 as PRW827.
Synthetic oligonucleotides RW165 (SEQ ID NO:207) and RW166
(SEQ ID NO:208) were annealed, cut with <u>Hinc</u>II and <u>Sty</u>I and
inserted between the <u>Eco</u>RV and <u>Sty</u>I sites of pRW827 to
generate pRW845.

RW165 (SEQ ID NO:207):   5' GTACAGGTCGACAAGCTTCCCGGGTATCGCG
                          ATATCCGTTAAGTTTGTATCGTAATGAATACTCAA
                          ATTCTAATACTCACTCTTGTGGCAGCCATTCACAC
                          AAATGCAGACAAAATCTGCCTTGGACATCAT 3'

RW166 (SEQ ID NO:208):   5' ATGATGTCCAAGGCAGATTTTGTCTGCATTTG
                          TGTGAATGGCTGCCACAAGAGTGAGTATTAGAATT
                          TGAGTATTCATTACGATACAAACTTAACGGATATC
                          GCGATACCCGGGAAGCTTGTCGACCTGTAC 3'

    Oligonucleotides RW165 (SEQ ID NO:207) and RW166 (SEQ
ID NO:208) link the 3' portion of the H6 promoter to the H7
gene. The 3' non-coding end of the H7 gene was removed by
isolating the linear product of an <u>Apa</u>LI digestion of
pRW845, recutting it with <u>Eco</u>RI, isolating the largest
fragment and annealing with synthetic oligonucleotides RW227
(SEQ ID NO:209) and RW228 (SEQ ID NO:210). The resulting
plasmid was pRW854.

RW227 (SEQ ID NO:209):   5' ATAACATGCGGTGCACCATTTGTATAT
                          AAGTTAACGAATTCCAAGTCAAGC 3'

RW228 (SEQ ID NO:210):   5' GCTTGACTTGGAATTCGTTAACTTATA
                          TACAAATGGTGCACCGCATGTTAT 3'

The stop codon of H7 in pRW854 is followed by an HpaI site.
The intermediate H6 promoted H7 construct in the de-ORFed F7
locus (described below) was generated by moving the pRW854
EcoRV-HpaI fragment into pRW858 which had been cut with
EcoRV and blunt-ended at its PstI site. Plasmid pRW858
(described below) contains the H6 promoter in an F7 de-ORFed
insertion plasmid.

     The plasmid pRW858 was constructed by insertion of an
850 bp SmaI/HpaI fragment, containing the H6 promoter linked
to a non-pertinent gene, into the SmaI site of pF7DO
described previously. The non-pertinent sequences were
excised by digestion of pRW858 with EcoRV (site 24 bp
upstream of the 3'-end of the H6 promoter) and PstI. The
3.5 kb resultant fragment was isolated and blunt-ended using
the Klenow fragment of the E. coli DNA polymerase in the
presence of 2mM dNTPs. This blunt-ended fragment was
ligated to a 1700 bp EcoRV/HpaI fragment derived from pRW854
(described previously). This EcoRV/HpaI fragment contains
the entire AIV HA (H7) gene juxtaposed 3' to the 3'-most 24
bp of the VV H6 promoter. The resultant plasmid was
designated pRW861.

     The 126 bp EH arm (defined previously) was lengthened
in pRW861 to increase the recombination frequency with
genomic TROVAC DNA. To accomplish this, a 575 bp AccI/SnaBI
fragment was derived from pRW 731.13 (defined previously).
The fragment was isolated and inserted between the AccI and
NaeI sites of pRW861. The resultant plasmid, containing an
EH arm of 725 bp and a HB arm of 404 bp flanking the AIV H7
gene, was designated as pRW869. Plasmid pRW869 therefore
consists of the H7 coding sequence linked at its 5' end to
the vaccinia virus H6 promoter. The left flanking arm
consists of 404 bp of TROVAC sequence and the right flanking
arm of 725 bp of TROVAC sequence which directs insertion to
the de-ORFed F7 locus.

     Development of TROVAC-Avian Influenza Virus
Recombinants. Insertion plasmids containing the avian
influenza virus HA coding sequences were individually
transfected into TROVAC infected primary CEF cells by using
the calcium phosphate precipitation method previously

described (Panicali et al., 1982; Piccini et al., 1987).
Positive plaques were selected on the basis of hybridization
to HA specific radiolabelled probes and subjected to
sequential rounds of plaque purification until a pure
population was achieved. One representative plaque was then
amplified to produce a stock virus. Plasmid pRW849 was used
in an *in vitro* recombination test to produce recombinant
TROVAC-AIH5 (vFP89) expressing the H5 hemagglutinin.
Plasmid pRW848 was used to produce recombinant TROVAC-AIH4
(vFP92) expressing the H4 hemagglutinin. Plasmid pRW869 was
used to produce recombinant TROVAC-AIH7 (vFP100) expressing
the H7 hemagglutinin.

   Immunofluorescence. In influenza virus infected cells,
the HA molecule is synthesized and glycosylated as a
precursor molecule at the rough endoplasmic reticulum.
During passage to the plasma membrane it undergoes extensive
post-translational modification culminating in proteolytic
cleavage into the disulphide linked HA_1 and HA_2 subunits and
insertion into the host cell membrane where it is
subsequently incorporated into mature viral envelopes. To
determine whether the HA molecules produced in cells
infected with the TROVAC-AIV recombinant viruses were
expressed on the cell surface, immunofluorescence studies
were performed. Indirect immunofluorescence was performed
as described (Taylor et al., 1990). Surface expression of
the H5 hemagglutinin in TROVAC-AIH5, H4 hemagglutinin in
TROVAC-AIH4 and H7 hemagglutinin in TROVAC-AIH7 was
confirmed by indirect immunofluorescence. Expression of the
H5 hemagglutinin was detected using a pool of monoclonal
antibodies specific for the H5HA. Expression of the H4HA
was analyzed using a goat monospecific anti-H4 serum.
Expression of the H7HA was analyzed using a H7 specific
monoclonal antibody preparation.

   Immunoprecipitation. It has been determined that the
sequence at and around the cleavage site of the
hemagglutinin molecule plays an important role in
determining viral virulence since cleavage of the
hemagglutinin polypeptide is necessary for virus particles
to be infectious. The hemagglutinin proteins of the

virulent H5 and H7 viruses possess more than one basic amino
acid at the carboxy terminus of HA1. It is thought that
this allows cellular proteases which recognize a series of
basic amino acids to cleave the hemagglutinin and allow the
infectious virus to spread both in vitro and in vivo. The
hemagglutinin molecules of H4 avirulent strains are not
cleaved in tissue culture unless exogenous trypsin is added.

In order to determine that the hemagglutinin molecules
expressed by the TROVAC recombinants were authentically
processed, immunoprecipitation experiments were performed as
described (Taylor et al., 1990) using the specific reagents
described above.

Immunoprecipitation analysis of the H5 hemagglutinin
expressed by TROVAC-AIH5 (vFP89) showed that the
glycoprotein is evident as the two cleavage products HA_1 and
HA_2 with approximate molecular weights of 44 and 23 kDa,
respectively. No such proteins were precipitated from
uninfected cells or cells infected with parental TROVAC.
Similarly immunoprecipitation analysis of the hemagglutinin
expressed by TROVAC-AIH7 (vFP100) showed specific
precipitation of the HA_2 cleavage product. The HA_1 cleavage
product was not recognized. No proteins were specifically
precipitated from uninfected CEF cells or TROVAC infected
CEF cells. In contrast, immunoprecipitation analysis of the
expression product of TROVAC-AIH4 (vFP92) showed expression
of only the precursor protein HA_0. This is in agreement
with the lack of cleavage of the hemagglutinins of avirulent
subtypes in tissue culture. No H4 specific proteins were
detected in uninfected CEF cells or cells infected with
TROVAC.

Example 23 - DEVELOPMENT OF A TRIPLE RECOMBINANT
                EXPRESSING THREE AVIAN INFLUENZA GENES

Plasmid Construction. Plasmid pRW849 has been
discussed previously and contains the H6 promoted avian
influenza H5 gene. This plasmid was used for the
development of vFP89. Plasmid pRW861 was an intermediate
plasmid, described previously used in the development of
vFP100. The plasmid contains the H6 promoted avian
influenza H7 gene. Plasmid pRW849 was digested with SmaI

and the resulting 1.9 kbp fragment from the 5' end of the H6
promoter through the H5 gene was inserted at the SmaI site
of pRW861 to produce pRW865. In order to insert the H4
coding sequence, plasmid pRW848 was utilized. Plasmid
pRW848 was used in the development of vFP92 and contains the
H6 promoted H4 gene (previously described). Plasmid pRW848
was digested with SmaI and a 1.9 kbp fragment containing the
H6 promoted H4 coding sequence was then inserted into pRW865
at the SmaI site 5' of the H6 promoted H5 sequence. The
resulting plasmid pRW872 therefore contains the H4, H5 and
H7 coding sequences in the F7 de-ORFed insertion plasmid.

In order to direct insertion of the genes to the de-
ORFed F8 locus, pRW872 was partially digested with SmaI, the
linear fragment isolated and recut with HindIII. The 5.7
kbp SmaI to HindIII pRW872 fragment containing all three H6
promoted avian influenza genes was blunt-ended and inserted
into pCEN100 which had been cut with HincII. Plasmid
pCEN100 is a de-ORFed F8 insertion vector containing
transcription and translation stop signals and multiple
insertion sites. Plasmid pCEN100 was generated as described
below. Synthetic oligonucleotides CE205 (SEQ ID NO:211) and
CE206 (SEQ ID NO:212) were annealed, phosphorylated and
inserted into the BamHI and HindIII sites of pCE72
(previously described) to form pCE72. A BglII to EcoRI
fragment from pCE72 was inserted into the BglII and EcoRI
sites of pJCA021 to form pCEN100.

CE205 (SEQ ID NO:211):   5' GATCAGAAAAACTAGCTAGCTAGTACGTAGTT
                          AACGTCGACCTGCAGAAGCTTCTAGCTAGCTAGTT
                          TTTAT 3'

CE206 (SEQ ID NO:212):   5' AGCTATAAAAACTAGCTAGCTAGAAGCTTCTG
                          CAGGTCGACGTTAACTACGTACTAGCTAGCTAGTT
                          TTTCT 3'

Plasmid pJCA021 was obtained by inserting a 4900 bp PvuII-
HindII fragment from pRW731-15 (previously described) into
the SmaI and HindII sites of pBSSKT.

The final insertion plasmid pRW874 had the three avian
influenza HA genes transcribed in the same direction as the
deleted F8 ORF. The left flanking arm of the plasmid
adjacent to the H4 gene consisted of 2350 bp of fowlpox

sequence. The right flanking arm adjacent to the H7 gene
consisted of 1700 bp of fowlpox sequence. A linear
representation of the plasmid is shown below.

      2350bp FP H6 H4HA H6 H5HA H6   H7HA   1700bp FP
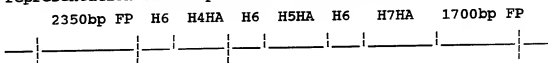
     Development of Recombinant vFP122. Plasmid pRW874 was
transfected into TROVAC infected primary CEF cells by using
the calcium phosphate precipitation method previously
described (Panicali et al., 1982; Piccini et al., 1987).
Positive plaques were selected on the basis of hybridization
to specific H4, H5 and H7 radiolabelled probes and subjected
to 5 sequential rounds of plaque purification until a pure
population was achieved. Surface expression of all three
glycoproteins was confirmed by plaque immunoscreen using
specific reagents previously described. Stability of
inserted genes was confirmed after two rounds of
amplification and the recombinant was designated as vFP122.

Example 24 - **COMPARISON OF THE LD_{50} OF ALVAC AND NYVAC
                  WITH VARIOUS VACCINIA VIRUS STRAINS**

     Mice. Male outbred Swiss Webster mice were purchased
from Taconic Farms (Germantown, NY) and maintained on mouse
chow and water *ad libitum* until use at 3 weeks of age
("normal" mice). Newborn outbred Swiss Webster mice were of
both sexes and were obtained following timed pregnancies
performed by Taconic Farms. All newborn mice used were
delivered within a two day period.

     Viruses. ALVAC was derived by plaque purification of a
canarypox virus population and was prepared in primary chick
embryo fibroblast cells (CEF). Following purification by
centrifugation over sucrose density gradients, ALVAC was
enumerated for plaque forming units in CEF cells. The WR(L)
variant of vaccinia virus was derived by selection of large
plaque phenotypes of WR (Panicali et al., 1981). The Wyeth
New York State Board of Health vaccine strain of vaccinia
virus was obtained from Pharmaceuticals Calf Lymph Type
vaccine Dryvax, control number 302001B. Copenhagen strain
vaccinia virus VC-2 was obtained from Institut Merieux,
France. Vaccinia virus strain NYVAC was derived from

Copenhagen VC-2. All vaccinia virus strains except the
Wyeth strain were cultivated in Vero African green monkey
kidney cells, purified by sucrose gradient density
centrifugation and enumerated for plaque forming units on
Vero cells. The Wyeth strain was grown in CEF cells and
enumerated in CEF cells.

Inoculations. Groups of 10 normal mice were inoculated
intracranially (ic) with 0.05 ml of one of several dilutions
of virus prepared by 10-fold serially diluting the stock
preparations in sterile phosphate-buffered saline. In some
instances, undiluted stock virus preparation was used for
inoculation.

Groups of 10 newborn mice, 1 to 2 days old, were
inoculated ic similarly to the normal mice except that an
injection volume of 0.03 ml was used.

All mice were observed daily for mortality for a period
of 14 days (newborn mice) or 21 days (normal mice) after
inoculation. Mice found dead the morning following
inoculation were excluded due to potential death by trauma.

The lethal dose required to produce mortality for 50%
of the experimental population (LD$_{50}$) was determined by the
proportional method of Reed and Muench.

Comparison of the LD$_{50}$ of ALVAC and NYVAC with Various
Vaccinia Virus Strains for Normal, Young Outbred Mice by the
ic Route. In young, normal mice, the virulence of NYVAC and
ALVAC were several orders of magnitude lower than the other
vaccinia virus strains tested (Table 28). NYVAC and ALVAC
were found to be over 3,000 times less virulent in normal
mice than the Wyeth strain; over 12,500 times less virulent
than the parental VC-2 strain; and over 63,000,000 times
less virulent than the WR(L) variant. These results would
suggest that NYVAC is highly attenuated compared to other
vaccinia strains, and that ALVAC is generally nonvirulent
for young mice when administered intracranially, although
both may cause mortality in mice at extremely high doses
(3.85x10^8 PFUs, ALVAC and 3x10^8 PFUs, NYVAC) by an
undetermined mechanism by this route of inoculation.

Comparison of the LD$_{50}$ of ALVAC and NYVAC with Various
Vaccinia Virus Strains for Newborn Outbred Mice by the ic

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Route. The relative virulence of 5 poxvirus strains for
normal, newborn mice was tested by titration in an
intracranial (ic) challenge model system (Table 29). With
mortality as the endpoint, LD_{50} values indicated that ALVAC
is over 100,000 times less virulent than the Wyeth vaccine
strain of vaccinia virus; over 200,000 times less virulent
than the Copenhagen VC-2 strain of vaccinia virus; and over
25,000,000 times less virulent than the WR-L variant of
vaccinia virus. Nonetheless, at the highest dose tested,
6.3×10^7 PFUs, 100% mortality resulted. Mortality rates of
33.3% were observed at 6.3×10^6 PFUs. The cause of death,
while not actually determined, was not likely of
toxicological or traumatic nature since the mean survival
time (MST) of mice of the highest dosage group
(approximately 6.3 LD_{50}) was 6.7 ± 1.5 days. When compared
to WR(L) at a challenge dose of 5 LD_{50}, wherein MST is 4.8 ±
0.6 days, the MST of ALVAC challenged mice was significantly
longer (P=0.001).

    Relative to NYVAC, Wyeth was found to be over 15,000
times more virulent; VC-2, greater than 35,000 times more
virulent; and WR(L), over 3,000,000 times more virulent.
Similar to ALVAC, the two highest doses of NYVAC, 6×10^8 and
6×10^7 PFUs, caused 100% mortality. However, the MST of mice
challenged with the highest dose, corresponding to 380 LD_{50},
was only 2 days (9 deaths on day 2 and 1 on day 4). In
contrast, all mice challenged with the highest dose of WR-L,
equivalent to 500 LD_{50}, survived to day 4.

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Table 28. Calculated 50% Lethal
          Dose for mice by
          various vaccinia
          virus strains and for
          canarypox virus
          (ALVAC) by the ic
          route.

POXVIRUS STRAIN	CALCULATED LD_{50} (PFUs)
WR(L)	2.5
VC-2	1.26×10^4
WYETH	5.00×10^4
NYVAC	1.58×10^8
ALVAC	1.58×10^8

Table 29. Calculated 50% Lethal
          Dose for newborn mice
          by various vaccinia
          virus strains and for
          canarypox virus
          (ALVAC) by the ic
          route.

POXVIRUS STRAIN	CALCULATED LD_{50} (PFUs)
WR(L)	0.4
VC-2	0.1
WYETH	1.6
NYVAC	1.58×10^6
ALVAC	1.00×10^7

Example 25 - GENERATION OF NYVAC-BASED RECOMBINANTS
EXPRESSING THE EHV-1 gB, gC AND gD
GLYCOPROTEINS HOMOLOGS

Expression of the EHV-1 gB glycoprotein was
accomplished by putting the EHV-1 gB homolog gene under the
control of the vaccinia virus I3L promoter. Expression of
the EHV-1 gC glycoprotein was accomplished by putting the
EHV-1 gC homolog gene under the control of the vaccinia
virus H6 promoter. Expression of the EHV-1 gD glycoprotein
was accomplished by putting the EHV-1 gD homolog gene under
the control of the entomopox virus 42K gene promoter.

Generation of vP1025 (gB and gC in ATI locus; gD in HA
locus).

Generation of donor plasmid pJCA042. The 430 bp 5'-
most region of the EHV-1 gB coding sequence was PCR-derived
using the plasmid pJCA011 (cassette H6-EHV-1 gB in ATI
locus) as template and oligonucleotides JCA156 (SEQ ID
NO:223) (5'-ATGTCCTCTGGTTGCCGTTCT-3') and JCA157 (SEQ ID
NO:224) (5'-GACGGTGGATCCGGTAGGCGG-3'), digested with BamHI
and kinased. This 430 bp fragment was fused to a 120 bp
PCR-derived fragment containing the I3L promoter element
obtained using plasmid pMP691 (I3L101RAB) as template and
oligonucleotides JCA158 (SEQ ID NO:225) (5'-
TTTTTCTAGACTGCAGCCCGGGACATCATGCAGTGGTTAAAC-3') and MP287
(SEQ ID NO:226) (5'-GATTAAACCTAAATAATTGT-3'). This 120 bp
fragment was digested with XbaI and kinased prior to be
ligated with the 430 bp 5'-most region of EHV-1 gB fragment.
The resulting plasmid was designated pJCA034. Sequences of
the I3L promoter, of the junction I3L-ATG and of the EHV-1
5'-most region were confirmed by direct sequencing of
pJCA034. Plasmid pJCA034 was digested with SmaI and BamHI
to excise the 550 bp SmaI-I3L-EHV-1 gB 5'-BamHI fragment
(A). Plasmid pMP665 (cassette H6-EHV-1 gB in COPCS system)
was digested with BamHI and XhoI to excise the 2530 bp
BamHI-EHV-1 gB 3' fragment (B). Fragments A and B were then
ligated together into vector pSD541VC (ATI deorfed locus)
digested with SmaI and XhoI to produce pJCA037. Plasmid
pJCA037 is the donor plasmid containing the cassette I3L-
EHV-1 gB in the ATI deorfed locus. Plasmid pJCA037 was
digested with SmaI and XhoI to isolate the 3050 bp SmaI-I3L-

EHV-1 gB-XhoI fragment (C). The 225 bp KpnI-EHV-1 gC 3'end
cleaned up-HindIII fragment was PCR-derived using plasmid
pVHAH6g13 (cassette H6-EHV-1 gC in HA deorfed locus) and
oligonucleotides JCA154 (SEQ ID NO:227) (5'-
TATAGCTGCATAATAGAG-3') and JCA163 (SEQ ID NO:228) (5'-
AATTAAGCTTGATATCACAAAAACTAAAAAGTCAGACTTCTTG-3'), digested
with KpnI and HindIII, and ligated into vector pBS-SK⁺
digested with KpnI and HindIII to produce pJCA033. Sequence
of the cloned PCR fragment was confirmed by direct
sequencing of pJCA033. Plasmid pJCA033 was digested with
KpnI and EcoRV to isolate the 220 bp KpnI-EHV-1 gC 3'end-
EcoRV fragment (D). Plasmid pVHAH6g13 was digested with
BglII and KpnI to isolate the 1330 bp BglII-H6-EHV-1 gC 5'-
KpnI fragment (E).

     Fragments C, D and E were finally ligated together into
vector pSD541VC digested with BglII and XhoI to produce
plasmid pJCA042. Plasmid pJCA042 is the donor plasmid to
insert the I3L-EHV-1 gB -- H6-EHV-1 gC double construction
in the NYVAC ATI deorfed locus. Plasmid pJCA042 was
linearized using NotI prior to IVR.

     In vitro recombination experiment was performed on Vero
cells using pJCA042 as the donor plasmid and vP866 (NYVAC)
as the rescue virus. Standard protocols were used to
identify and purify the recombinant virus (Piccini et al.,
1987). The NYVAC-based recombinant containing the EHV-1 gB
and gC genes in the ATI deorfed locus was designated vP956.

     Generation of Donor Plasmid pJCA064. Plasmid
pEHV1BamHID (containing the EHV-1 BamHI D fragment) was
digested with HindIII to excise the 1240 bp HindIII-HindIII
containing the entire EHV-1 gD coding sequence but the 15
5'-most bp. The 1240 bp HindIII-HindIII fragment was blunt-
ended with Klenow polymerase and ligated into vector
pCOPCS657 digested with SmaI and phosphatased. The
resulting plasmid was designated pJCA006. Plasmid pJCA006
was digested with BglII and HindIII to excise the 1500 bp
HindIII-H6--EHV-1 gD-BglII fragment. This fragment was
ligated into vector pIBI24 digested with BamHI and HindIII
to produce plasmid pEHV1gp50a. Plasmid pEHV1gp50a was
digested with EcoRV and NcoI which are both unique sites to

excise the 4100 bp fragment. This fragment was ligated with
a synthetic double strand oligonucleotide obtained by
hybridization between oligonucleotides JCA052 (SEQ ID
NO:229) (5'-ATCCGTTAAGTTTGTATCGTAATGTCTACCTTCAAGCTTATGA
TGGATGGACGTTTGGTTTTTGC-3') and JCA053 (SEQ ID NO:230) (5'-
CATGGCAAAAACCAAACGTCCATCCATCATAAGCTTGAAGGTAGACATTACGATACAAAC
TTAAGCGAT-3'). The resulting plasmid was designated
pgp50a3-2. The 490 bp EcoRI-EHV-1 gD 3'end cleaned up –HpaI
was PCR-derived using plasmid pJCA006 as template and
oligonucleotides JCA041 (SEQ ID NO:231) (5'-TGTGTGA
TGAGAGATCAG-3') and JCA099 (SEQ ID NO:232) (5'-AACTC
GAGTTAACAAAAATTACGGAAGCTGGGTATATTTAACAT-3'). Plasmid
pgp50a3-2 was digested with EcoRI and partially digested
with HindIII to excise the 850 bp HindIII-H6-EHV-1 gD 5'-
EcoRI fragment. This fragment was ligated with the 490 bp
EcoRI-HpaI fragment into vector pBS-SK⁺ digested with
HindIII and SmaI to produce plasmid pJCA020. Plasmid
pJCA020 contains the cassette H6-EHV-1 gD.

The 720 bp 5'-most region of the EHV-1 gD coding
sequence was PCR-derived using plasmid pJCA020 as template
and oligonucleotides JCA044 (SEQ ID NO:233) (5'-CTCTAT
GACCTCATCCAC-3') and JCA165 (SEQ ID NO:234) (5'-ATGTCTA
CCTTCAAGCTTATG-3'). This fragment was digested with EcoRI
and kinased. The 107 bp 42K promoter element was PCR-
derived using plasmid pAM12 as template and oligonucleotides
RG286 (SEQ ID NO:235) (5'-TTTATATTGTAATTATA-3') and JCA164
(SEQ ID NO:236) (5'-TTTGGATCCGTTAACTCAAAAAAATAAATG-3').
This fragment was digested with BamHI, kinased, and ligated
with the 720 bp ATG-EHV-1 gD 5'-EcoRI fragment into vector
pBS-SK⁺ digested with BamHI and EcoRI to produce plasmid
pJCA035. Sequences of the 42K promoter, of the junction
42K-EHV-1 gD and of the EHV-1 gD 5' portion were confirmed
by direct sequencing of pJCA035.

Plasmid pJCA035 was digested with BamHI and EcoRI to
isolate the 830 bp BamHI-42K-EHV-1 gD 5'portion-EcoRI
fragment (F). Plasmid pJCA020 was digested with EcoRI and
XbaI to isolate the 500 bp EcoRI-EHV-1 gD 3'end cleaned up-
XbaI fragment (G). Fragments F and G were then ligated
together into vector pBS-SK⁺ digested with BamHI and XbaI to

produce plasmid pJCA038. Plasmid pJCA038 is containing the
cassette 42K-EHV-1 gD into vector pBS-SK⁺. Plasmid pJCA038
was digested with BamHI and HpaI to isolate the 1340 bp
HpaI-42K-EHV-1 gD-BamHI fragment. This fragment was ligated
into plasmid pSD544 (HA deorfed locus) digested with BamHI
and SmaI to produce plasmid pJCA064. Plasmid pJCA064 is the
donor plasmid to insert the cassette 42K-EHV-1 gD into the
NYVAC HA deorfed locus. Plasmid pJCA064 was linearized
using NotI prior to IVR.

In vitro experiment was performed on Vero cells using
pJCA064 as the donor plasmid and recombinant vaccinia virus
vP956 (NYVAC background) as the rescue virus. This was
performed with standard procedures (Piccini et al., 1987).
The NYVAC-based recombinant containing the EHV-1 gB and gC
genes in the ATI deorfed locus and the EHV-1 gD gene in the
HA deorfed locus was designated vP1025.

Generation of donor plasmid pJCA043. The 220 bp
HindIII-EHV-1 gB 3'-most region was PCR-derived using
plasmid pJCA011 and oligonucleotides JCA159 (SEQ ID NO:237)
(5'-AGGCCAAGCTTGAAGAGGCTC-3') and JCA160 (SEQ ID NO:238)
(5'-AAAGGATCCGTTAACACAAAAATTAAACCATTTTTTCATT-3'). This
fragment was digested with BamHI and HindIII and ligated
into vector pBS-SK⁺ digested with BamHI and HindIII to
produce plasmid pJCA036. Sequence of the EHV-1 gB 3'-most
region PCR fragment was confirmed by direct sequencing of
pJCA036.

Plasmid pJCA033 was digested with EcoRV and KpnI to
isolate the KpnI-EHV-1 gC 3'-most region-EcoRV fragment (H).
Plasmid pJCA038 was digested with BamHI and HpaI to isolate
the 1360 bp HpaI-42K-EHV-1 gD -BamHI fragment (I). Plasmid
pVHAH6g13 was digested with KpnI and XhoI to isolate the 900
bp XhoI-EHV-1 gC central portion-KpnI fragment (J).
Fragments H, I and J were then ligated together into vector
pBS-SK⁺ digested with BamHI and XhoI to produce plasmid
pJCA041.

Plasmid pJCA034 was digested with HindIII and XhoI to
isolate the 5900 bp linearized vector XhoI-pBS-SK⁺-I3L-EHV-1
gB -HindIII fragment (K). Plasmid pJCA036 was digested with
BamHI and HindIII to isolate the 220 bp HindIII-EHV-1 gB 3--

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most region-BamHI fragment (L). Plasmid pVHAH6g13 was
digested with BglII and XhoI to isolate the 440 bp BglII-H6-
EHV-1 gC 5'portion-XhoI fragment (M). Fragments K, L and M
were then ligated together to produce plasmid pJCA040.

Plasmid pJCA040 was digested with SmaI and XhoI to
isolate the 3550 bp SmaI-I3L-EHV-1 gB -- H6-EHV-1 gC
5'portion-XhoI fragment (N). Plasmid pJCA041 was digested
with BamHI and XhoI to isolate the 2460 bp XhoI-EHV-1 gC
3'portion -- 42K-EHV-1 gD -BamHI fragment (O). Fragments N
and O were finally ligated together into plasmid pSD541VC
(NYVAC ATI deorfed locus) digested with BglII and SmaI to
produce plasmid pJCA043. Plasmid pJCA043 is the donor
plasmid to insert the I3L-EHV-1 gB -- H6-EHV-1 gC -- 42K-
EHV-1 gD triple construction in the NYVAC ATI deorfed locus.
Plasmid pJCA043 was linearized using NotI prior to IVR.

In vitro experiment was performed on primary chick
embryo fibroblasts using pJCA043 as the donor plasmid and
vP866 (NYVAC) as the rescue virus. Standard procedures were
used to identify and purify the generated recombinant
(Piccini et al., 1987). The NYVAC-based recombinant
containing the EHV-1 gB, gC and gD genes in the ATI deorfed
locus was designated vP1043.

**Example 26 -   GENERATION OF ALVAC-BASED RECOMBINANTS
                  EXPRESSING THE EHV-1 gB, gC AND gD
                  GLYCOPROTEINS HOMOLOGS**

Generation of donor plasmid pJCA049. Plasmid pJCA040
was digested with SmaI and XhoI to isolate the 3550 bp SmaI-
I3L-EHV-1 gB -- H6-EHV-1 gC 5'portion-XhoI fragment (A).
Plasmid pJCA041 was digested with BamHI and XhoI to isolate
the 2460 bp XhoI-EHV-1 gC 3'portion -- 42K-EHV-1 gD -BamHI
fragment (B). Fragments A and B were ligated together into
plasmid pSPVQC3L digested with BamHI and SmaI to produce
plasmid pJCA049. Plasmid pJCA049 is the donor plasmid to
insert the I3L-EHV-1 gB -- H6-EHV-1 gC -- 42K-EHV-1 gD
triple construction in the ALVAC C3 deorfed locus. Plasmid
pJCA049 was linearized using NotI prior to IVR.

In vitro experiment was performed on primary chick
embryo fibroblasts using pJCA049 as the donor plasmid and
CPpp (ALVAC) as the rescue virus. Standard procedures were
followed to identify and purify the generated recombinant

(Piccini et al., 1987). The ALVAC-based recombinant
containing the EHV-1 gB, gC and gD genes in the C3 deorfed
locus was designated vCP132.

**Example 27 - EXPRESSION ANALYSIS OF THE NYVAC- AND ALVAC-
BASED EQUINE HERPESVIRUS TYPE 1 TRIPLE
RECOMBINANTS**

Immunofluorescence assays were performed as described
previously (Taylor et al., 1990) using monoclonal antibodies
specific to EHV-1 gB (16G5 or 3F6), EHV-1 gC (14H7) and EHV-
1 gD (20C4). All anti-EHV-1 monoclonals were obtained from
George Allen (Department of Veterinary Science, University
of Kentucky, Lexington, Kentucky, 40546-0076). Expression
of all three EHV-1 specific products was detectable
internally in cells infected with either vP1025, vP1043 or
vCP132. Only the EHV-1 gC glycoprotein was well expressed
on the surface of infected cells. Surface expression for
EHV-1 gB glycoprotein was much weaker and surface expression
of EHV-1 gD glycoprotein was questionable.

Immunoprecipitations were done using the same
monoclonal antibodies to determine the authenticity of the
expressed EHV-1 gB, gC and gD gene products. Monoclonal 3F6
specific for EHV-1 gB glycoprotein precipitated proteins
with apparent molecular masses on an SDS-PAGE gel system of
138 kDa, 70 kDa and 54 kDa from lysates derived from cells
infected with the recombinant viruses vP956, vP1025, vP1043
or vCP132. No protein was precipitated from lysates derived
from uninfected cells or from either parental virus (NYVAC
and ALVAC) infected cells. Monoclonal 14H7 specific for
EHV-1 gC glycoprotein precipitated a glycoprotein with an
apparent molecular mass of 90 kDa from lysates derived from
cells infected either with vP956, vP1025 or vP1043. The
EHV-1 gC glycoprotein expressed by recombinant vCP132 has an
apparent molecular mass slightly smaller (about 2 kDa less)
than that expressed by recombinants vP956, vP1025 or vP1043.
Monoclonal antibody 20C4 specific for EHV-1 gD glycoprotein
precipitated a glycoprotein with an apparent molecular mass
of 55 kDa from lysates derived from cells infected with
vP1025, vP1043 or vCP132.

Immunoprecipitations were also done using a rabbit
anti-EHV-1 hyperimmune serum obtained from G. Allen. This

serum precipitated all three EHV-1 products from lysates
derived from cells infected either with vP1025, vP1043 or
vCP132.

**Example 28   -   PROTECTION DATA OBTAINED USING THE HAMSTER
                   CHALLENGE MODEL**

Challenge experiments (hamster model) have been done at
Rhone-Merieux (Lyon, France) to assess the relative level of
protection induced by poxvirus EHV-1 recombinants vP956,
vP1025 and vCP132. Hamsters were vaccinated on day 0 and
boosted on day 14 with various dilutions of the EHV-1
recombinants. All immunized and control animals were
challenged on day 28 with a hamster-adapted EHV-1 challenge
strain. Final count of dead animals was made on day 35 (7
days post challenge). Results of the challenge experiment
are shown below in Table 30:

Table 30

Recombinant	EHV-1 genes	Dose TCID50 $\log_{10}$ /dead/total		
vP956	gB + gC	7.6	5.6	3.6
		0/4	3/4	2/4
vP1025	gB + gC + gD	7.8	5.8	3.8
		2/4	3/4	3/4
vCP132	gB + gC + gD	8.8	6.8	4.8
		0/4	0/4	2/4
Control	none	4/4		

Example 29 - DURATION OF IMMUNITY STUDIES IN DOGS

The aim of this study was to determine how long a
protective immune response would be maintained in dogs after
a single inoculation with ALVAC-RG (vCP65). Forty-one
beagle dogs of 8 months of age which were free of anti-
rabies antibody were inoculated with one dose of 6.7 $\log_{10}$
$TCID_{50}$ of ALVAC-RG by the subcutaneous route. Dogs were
bled on day 0 and at 1, 2, 3, 6 and 12 months after
vaccination and sera assayed for the presence of anti-rabies
antibody using the RFFI test. All animals were monitored
for side-effects of vaccination.

At 6 months post-vaccination, 5 dogs were challenged by
intra-muscular inoculation of the virulent NYGS strain of
rabies virus. Animals received $10^{3.4}$ 50% mouse lethal doses
in the temporal muscle. Three uninoculated control animals
received the same inoculation. A second group of 11
vaccinated dogs and 3 non-vaccinated control dogs were
challenged in an identical manner at 12 months post-
vaccination. The serological results and results of
challenge at 6 and 12 months are shown below in Table 31.

None of the dogs vaccinated with ALVAC-RG (vCP65)
exhibited adverse reaction to vaccination. All dogs
vaccinated with ALVAC-RG (vCP65) demonstrated the induction
of rabies virus neutralizing antibody by 7 days post-
vaccination. Maximal titers were achieved between 14 and 28
days post-vaccination after which titers decreased. At the
time of challenge at 6 or 12 months post-vaccination titers
were low and in some animals, approaching zero. Despite the
low titers, all animals survived a lethal rabies challenge
in which unvaccinated control dogs succumbed. RFFI titers
of animals that survived challenge at 6 months post-
vaccination were assessed at 8 months (2 months post-
challenge). The serum titers in these animals were 7.4,
7.4, 2.3, 1.8 and 7.4. International Units. These elevated
and maintained levels of rabies neutralizing antibody
indicate that animals were efficiently primed by the initial
single inoculation. The experiment is on-going and the
remaining animals will be challenged at 2 or 3 years
following vaccination; however, to date, the experiment is

successful and illustrates the utility of the present
invention.

Table 31. Duration of immunity study in dogs vaccinated with ALVAC-RG: Results of challenge at 6 and 12 months

| Treatment | Dog # | RFPI Titer in International Units | | Survival or Day of Death |
		Maximum at day 28	Day of Challenge	
SIX MONTH CHALLENGE[a]				
ALVAC-RG[b]	N2T90	7.45	0.23	S
	N3V57	14.80	0.23	S
	N4V49	7.45	0.29	S
	N5W22	8.70	0.59	S
	N7W22	7.45	0.18	S
Non-vaccinated	194	0.47	0.07	D (16)
	195	0.07	0.07	D (24)
	198	0.47	0.07	D (17)
TWELVE MONTH CHALLENGE				
ALVAC-RG	N1V9	7.45	0.09	S
	N3V9	7.45	0.15	S
	N2V9	3.73	0.07	S
	N3W22	9.78	0.05	S
	N4V9	7.45	0.15	S
	N1W38	18.70	0.15	S
	N8W22	2.35	0.15	S
	N8V14	7.45	0.38	S
	N7V14	3.73	0.15	S
	N1R45	7.45	0.12	S
	N9V14	0.94	0.15	S

<u>Table 31</u> (Continued)

Treatment	Dog #	RFFI Titer in International Units		Survival or Day of Death
		Maximum at day 28	Day of Challenge	
Non-vaccinated				
	N10V14	0.74	0.05	D (14)
	N14V14	0.59	0.05	D (13)
	N5R54	0.74	0.05	D (14)

a: Animals were challenged at 6 and 12 months post-vaccination with a dose of $10^{3.4}$ 50% mouse lethal doses of NYGS strain of rabies virus in the temporal muscle.

b: Dogs received one inoculation of 6.7 $\log_{10}$ $TCID_{50}$ by the subcutaneous route.

S: Dog survived challenge.

D: Dog died on (day).

**Example 30   -   EXPRESSION OF BOVINE HERPESVIRUS TYPE 1 BHV1
GENES IN NYVAC**

Generation of NYVAC/BHV1 gIV recombinant. A plasmid,
pBHVgIV, was obtained from Rhone Merieux. This plasmid
contains the BHV1 gIV gene (Straub strain), encoded on a 3.9
kb PstI fragment, cloned into the PstI site of pBS-SK$^+$. The
gIV gene (Tikoo et al., J. Virol. (1990) **64**:5132) from this
plasmid was cloned between vaccinia virus flanking arms.
This was accomplished by cloning the 2,000 bp PstI-XhoI
fragment of pBHVgIV, containing the gIV gene, into the PstI-
XhoI site of pSD542 (defined in Example 32). The plasmid
generated by this manipulation is called pBHV1.

The 3'-end of the π promoter was then cloned upstream
of the gIV gene. This was accomplished by cloning the
oligonucleotides, BHVL7 (SEQ ID NO:239) (5'-TCGAGCTTAA
GTCTTATTAATATGCAAGGGCCGACATTGGCCGTGCTGGGCGCGCTGCTCGCCGTTGCGG
TGAGCTTGCCTACACCCGCGCCGC-3') and BHVL8 (SEQ ID NO:240)
(5'-GGCGCGGGTGTAGGCAAGCTCACCGCAACGGCGAGCAGCGCGCCCAGCAC
GGCCAATGTCGGCCCTTGCATATTAATAAGACTTAAGC-3'), encoding the 3'-
end of the π promoter and the 5'-end of the gIV gene, into
the 5,500 bp partial SstII-XhoI fragment of pBHV1. The
plasmid generated by this manipulation is called pBHV3.

Extraneous 3'-noncoding sequence was then eliminated.
This was accomplished by cloning the oligonucleotides, BHVL5
(SEQ ID NO:241) (5'-GGGTGACTGCA-3') and BHVL6 (SEQ ID
NO:242) (5'-GTCACCC-3'), into the 5,200 bp partial SmaI-PstI
fragment of pBHV3. The plasmid generated by this
manipulation is called pBHV4.

Extraneous linker sequence was then eliminated. This
was accomplished by ligating the 5,200 bp PstI fragment of
pBHV4. The plasmid generated by this manipulation is called
pBHV5.

The 5'-end of the π promoter was then cloned into
pBHV5. This was accomplished by cloning the 130 bp AflII-
XhoI fragment of pPI4, containing the 5'-end of the π
promoter, into the 5,200 bp AflII-XhoI fragment of pBHV5.
The plasmid generated by this manipulation is called pBHV6.

pBHV6 was used in in vitro recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP1051.

Immunoprecipitation analysis was performed to determine
whether vP1051 expresses an authentic BHV1 gIV glycoprotein.
Vero cell monolayers were either mock infected, infected
with NYVAC or infected with vP1051 at an m.o.i. of 10
PFU/cell. Following an hour adsorption period, the inoculum
was aspirated and the cells were overlayed with 2 mls of
modified Eagle's medium (minus methionine) containing 2%
fetal bovine serum and [^{35}S]-methionine (20 μCi/ml). Cells
were harvested at 7 hrs post-infection by the addition of 1
ml 3X buffer A (3% NP-40, 30mM Tris (pH7.4), 3mM EDTA, 0.03%
Na Azide and 0.6 mg/ml PMSF) and 50 mls aprotinin, with
subsequent scraping of the cell monolayers.

Lysates were then analyzed for BHV1 gIV expression
using the BHV1 gIV-specific monoclonal antibody, 3402
(obtained from Dr. Geoffrey Letchworth, U. of Wisconsin,
Madison, WI). This was accomplished by the following
procedure: rat anti-mouse sera was bound to protein-A
sepharose at room temperature for 4 hours. After washing
the material 5X with 1X buffer A, the protein A-sepharose
bound rat anti-mouse antibody was bound to the gIV-specific
monoclonal antibody, 3402. The lysates, meanwhile, were
precleared by incubating normal mouse sera and the protein
A-sepharose bound rat anti-mouse antibody overnight at 4°C.
After washing this material 5X with 1X buffer A, the BHV1
gIV-specific monoclonal antibody, rat anti-mouse, protein A-
sepharose conjugate was added to the lysate and incubated
overnight at 4°C. After washing the samples 4X with 1X
buffer A and 2X with a LiCl$_2$/urea buffer, the precipitated
proteins were dissociated from the immune complexes by the
addition of 2X Laemmli's buffer (125 mM Tris (pH6.8), 4%
SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5
min. Proteins were then fractionated on a 10% Dreyfuss gel
system (Dreyfuss et al., 1984), fixed and treated with 1M
Na-salicylate for fluorography.

The BHV1 gIV-specific monoclonal antibody, 3402,
specifically precipitated the BHV1 gIV glycoprotein from
vP1051 infected cells, but did not precipitate BHV1-specific
proteins from NYVAC or mock infected cells.

Generation of NYVAC/BHV1 gI and gIV Recombinant. A

plasmid, pBHVgIV, containing the BHV1 gIV gene, was obtained
from Rhone Merieux. The gIV gene from this plasmid was
cloned between vaccinia virus flanking arms. This was
accomplished by cloning the 2,000 bp PstI-XhoI fragment of
pBHVgIV, containing the gIV gene, into the PstI-XhoI site of
pSD542. The plasmid generated by this manipulation is
called pBHV1.

The 3'-end of the π promoter was then cloned upstream
of the gIV gene. This was accomplished by cloning the
oligonucleotides, BHVL7 (SEQ ID NO:239) and BHVL8 (SEQ ID
NO:240), encoding the 3'-end of the π promoter and the 5'-
end of the gIV gene, into the 5,500 bp partial SstII-XhoI
fragment of pBHV1. The plasmid generated by this
manipulation is called pBHV3.

Extraneous 3'-noncoding sequence was then eliminated.
This was accomplished by cloning the oligonucleotides, BHVL5
(SEQ ID NO:241) and BHVL6 (SEQ ID NO:242), into the 5,200 bp
partial SmaI-PstI fragment of pBHV3. The plasmid generated
by this manipulation is called pBHV4.

Extraneous linker sequence was then eliminated. This
was accomplished by ligating the 5,200 bp PstI fragment of
pBHV4. The plasmid generated by this manipulation is called
pBHV5.

The 5'-end of the π promoter was then cloned into
pBHV5. This was accomplished by cloning the 130 bp AflII-
XhoI fragment of pPI4, containing the 5'-end of the π
promoter, into the 5,200 bp AflII-XhoI fragment of pBHV5.
The plasmid generated by this manipulation is called pBHV6.

The BHV1 gI gene was then cloned into pBHV6. This was
accomplished by cloning the 2,900 bp BglII fragment of
pBHV8, containing the H6-promoted gI gene, into the BglII
site of pBHV6. The plasmid generated by this manipulation
is called pBHV9.

pBHV8 was generated by the following procedure: a
plasmid, pIBRS6, was received from Rhone Merieux. The
plasmid contains a 6.6 kb SalI fragment, containing the BHV1
gI gene (Straub strain). The 5'-end of the gI gene
(Whitbeck et al., J. Virol. (1988) 62:3319) was cloned
downstream of the H6 promoter and between vaccinia virus HA

flanking arms. This was accomplished by cloning the 540 bp
SalI-PstI fragment of pIBRS6 into the 4,400 bp SalI-PstI
fragment of pGD5. The plasmid generated by this
manipulation is called pIBR2.

The initiation codon of the H6 promoter was then
aligned with the initiation codon of the gI gene. This was
accomplished by cloning the oligonucleotides, IBRL1 (SEQ ID
NO:243) (5'-ATCCGTTAAGTTTGTATCGTAATGGCCGCTCGCGGCGGTGCTGAA
CGCGCCGC-3') and IBRL2 (SEQ ID NO:244) (5'-GGCGCGTTCAGCA
CCGCCGCGAGCGGCCATTACGATACAAACTTAACGGAT-3'), into the 3,800
bp NruI-SstII fragment of pIBR2. The plasmid generated by
this manipulation is called pIBR4.

An NcoI site, necessary for future manipulations, was
then generated downstream from the gI sequence. This was
accomplished by cloning the oligonucleotides, IBRL3 (SEQ ID
NO:245) (5'-CCATGGTTTAATGCA-3') and IBRL4 (SEQ ID NO:246)
(5'-TTAAACCATGGTGCA-3'), into the PstI site of pIBR4. The
plasmid generated by this manipulation is called pIBR5.

Additional gI sequence was then cloned into pIBR5.
This was accomplished by cloning the 1,740 bp Tth111I-NcoI
fragment of pIBRS6 into the 3,700 bp Tth111I-NcoI fragment
of pIBR5. The plasmid generated by this manipulation is
called pIBR7.

A BglII site, necessary for future manipulations, was
then generated downstream from the gI sequence. This was
accomplished by cloning the oligonucleotides, IBRL5 (SEQ ID
NO:247) (5'-CATGGTTTAAGATCTC-3') and IBRL6 (SEQ ID NO:248)
(5'-CATGGAGATCTTAAAC-3'), into the NcoI site of pIBR7. The
plasmid generated by this manipulation is called pIBR8.

The 3'-end of the gI gene was then cloned into pIBR8.
This was accomplished by cloning the 2,285 bp StuI fragment
of pIBRS6 into the E. coli DNA polymerase I (Klenow
fragment) filled-in 4,300 bp StuI-BglII (partial) fragment
of pIBR8. The plasmid generated by this manipulation is
called pIBR20.

The H6-promoted BHV1 gI gene was then moved to a
vaccinia virus donor plasmid. This was accomplished by
cloning the E. coli DNA polymerase I (Klenow fragment)
filled-in 2,900 bp BglII-NcoI (partial) fragment of pIBR20

into the SmaI site of pSD542. This places the H6-promoted
gI gene between tk flanking arms. The plasmid generated by
this manipulation is called pIBR22.

A BglII site was then created upstream from the H6
promoter. This was accomplished by cloning the 2,800 bp
HindIII-EcoRV fragment of pIBR22 into the 3,500 bp HindIII-
EcoRV fragment of pGD3. (pGD3 is a plasmid that contains a
BglII site upstream from an H6-promoted herpes simplex virus
type 2 (HSV2) gD gene. This manipulation repalces the
HSV2gD sequence with the BHVIgI gene, thereby creating a
BglII site upstream from the H6-promoted gI gene). The
plasmid generated by this manipulation is called pBHV8.

pBHV8 was used in in vitro recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP1074.

Immunoprecipitation analysis was performed to determine
whether vP1074 expresses authentic BHV1 gI and gIV
glycoproteins. Vero cell monolayers were either mock
infected, infected with NYVAC or infected with vP1074 at an
m.o.i. of 10 PFU/cell. Following an hour adsorption period,
the inoculum was aspirated and the cells were overlayed with
2 mls of modified Eagle's medium (minus methionine)
containing 2% fetal bovine serum and [^{35}S]-methionine (20
μCi/ml). Cells were harvested at 7 hrs post-infection by
the addition of 1 ml 3X buffer A (3% NP-40, 30mM Tris
(pH7.4), 3mM EDTA, 0.03% Na Azide and 0.6 mg/ml PMSF) and 50
mls aprotinin, with subsequent scraping of the cell
monolayers.

Lysates were then analyzed for BHV1 gI and gIV
expression using the BHV1 gI-specific monoclonal antibody,
5106, and the gIV-specific monoclonal antibody, 3402
(obtained from Dr. Geoffrey Letchworth, U. of Wisconsin,
Madison, WI). This was accomplished by the following
procedure: rat anti-mouse sera was bound to protein-A
sepharose at room temperature for 4 hours. After washing
the material 5X with 1X buffer A, the protein A-sepharose
bound rat anti-mouse antibody was bound to the gI-specific
monoclonal antibody and the gIV-specific monoclonal
antibody. The lysates, meanwhile, were precleared by
incubating normal mouse sera and the protein A-sepharose

bound rat anti-mouse antibody overnight at 4°C. After
washing this material 5X with 1X buffer A, the gI or gIV-
specific monoclonal antibody, rat anti-mouse, protein A-
sepharose conjugate was added to the lysate and incubated
overnight at 4°C. After washing the samples 4X with 1X
buffer A and 2X with a LiCl$_2$/urea buffer, the precipitated
proteins were dissociated from the immune complexes by the
addition of 2X Laemmli's buffer (125 mM Tris (pH6.8), 4%
SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5
min. Proteins were then fractionated on a 10% Dreyfuss gel
system (Dreyfuss et al., 1984), fixed and treated with 1M
Na-salicylate for fluorography.

The BHV1 gI and gIV-specific monoclonal antibodies,
5106 and 3402, specifically precipitated the BHV1 gI and gIV
glycoproteins from vP1074 infected cells, but did not
precipitate BHV1-specific proteins from mock or NYVAC
infected cells.

Generation of NYVAC/BHV1 gIII recombinant. A plasmid,
pBHVgIII, was obtained from Rhone Merieux. This plasmid
contains the BHV1 gIII gene (Straub strain), encoded on a
3.4 kb BamHI/HindIII fragment cloned into the BamHI/HindIII
site of pBSK⁺. The gIII gene (Fitzpatrick et al., Virology
(1989) 173:146) from this plasmid was cloned between
vaccinia virus flanking arms. This was accompslished by
cloning the 1,000 bp NcoI-XhoI fragment of pBHVgIII,
containing the 5'-end of the gIII gene, and the
oligonucleotides, BHVL1 (SEQ ID NO:249) (5'-GATCCTGAGAT
AAAGTGAAAATATATATCATTATATTACAAAGTACAATTATTTAGGTTTAAT-3') and
BHVL2 (SEQ ID NO:250) (5'-CATGATTAAACCTAAATAATTGT
ACTTTGTAATATAATGATATATATTTTCACTTTATCTCAG-3'), encoding the
I3L promoter, into the BamHI-XhoI site of pSD544. The
plasmid generated by this manipulation is called pBHV2.

The 3'-end of the gIII gene was then cloned into pBHV2.
This was accomplished by cloning the oligonucleotides,
BHVL15 (SEQ ID NO:251) (5'-TCGAGCCCGGGTAATCCAACCCGGTC
TTACTCGCGCTCGCGCCCTCGGCTCCGCGCCCTAGG-3') and BHVL16 (SEQ ID
NO:252) (5'-GTACCCTAGGGCGCGGAGCCGAGGGCGCGAGCGCGAG
TAAGACCGGGTTGGATTACCCGGGC-3'), encoding the 3'-end of the
gIII gene, into the 4,700 bp XhoI-Asp718 fragment of pBHV2.

The plasmid generated by this manipulation is called pBHV7.

The rest of the gIII gene was then cloned into pBHV7.
This was accomplished by cloning the 500 bp partial SmaI-
XhoI fragment of pBHVgIII, containing an interior portion of
the gIII gene, into the 4,750 bp partial SmaI-XhoI fragment
of pBHV7. The plasmid generated by this manipulation is
called pBHV10.

pBHV10 was used in *in vitro* recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP1073.

Immunoprecipitation analysis was performed to determine
whether vP1073 expresses an authentic BHV1 gIII
glycoprotein. Vero cell monolayers were either mock
infected, infected with NYVAC or infected with vP1073 at an
m.o.i. of 10 PFU/cell. Following an hour adsorption period,
the inoculum was aspirated and the cells were overlayed with
2 mls of modified Eagle's medium (minus methionine)
containing 2% fetal bovine serum and [^{35}S]-methionine (20
μCi/ml). Cells were harvested at 7 hrs post-infection by
the addition of 1 ml 3X buffer A (3% NP-40, 30mM Tris
(pH7.4), 3mM EDTA, 0.03% Na Azide and 0.6 mg/ml PMSF) and 50
mls aprotinin, with subsequent scraping of the cell
monolayers.

Lysates were then analyzed for BHV1 gIII expression
using the BHV1 gIII-specific monoclonal antibody, 1507
(obtained from Dr. Geoffrey Letchworth, U. of Wisconsin,
Madison, WI). This was accomplished by the following
procedure: rat anti-mouse sera was bound to protein-A
sepharose at room temperature for 4 hours. After washing
the material 5X with 1X buffer A, the protein A-sepharose
bound rat anti-mouse antibody was bound to the gIII-specific
monoclonal antibody, 1507. The lysates, meanwhile, were
precleared by incubating normal mouse sera and the protein
A-sepharose bound rat anti-mouse antibody overnight at 4°C.
After washing this material 5X with 1X buffer A, the gIII-
specific monoclonal antibody, rat anti-mouse, protein A-
sepharose conjugate was added to the lysate and incubated
overnight at 4°C. After washing the samples 4X with 1X
buffer A and 2X with a LiCl$_2$/urea buffer, the precipitated
proteins were dissociated from the immune complexes by the

addition of 2X Laemmli's buffer (125 mM Tris (pH6.8), 4%
SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5
min. Proteins were then fractionated on a 10% Dreyfuss gel
system (Dreyfuss et al., 1984), fixed and treated with 1M
Na-salicylate for fluorography.

The BHV1 gIII-specific monoclonal antibody, 1507,
specifically precipitated the BHV1 gIII glycoprotein from
vP1073 infected cells, but did not precipitate BHV1-specific
proteins from mock or NYVAC infected cells.

Generation of NYVAC/BHV1 gIII AND gIV recombinant. A
plasmid, pBHVgIV, containing the BHV1 gIV gene, was obtained
from Rhone Merieux. The gIV gene from this plasmid was
cloned between vaccinia virus flanking arms. This was
accomplished by cloning the 2,000 bp PstI-XhoI fragment of
pBHVgIV, containing the gIV gene, into the PstI-XhoI site of
pSD542. The plasmid generated by this manipulation is
called pBHV1.

The 3'-end of the π promoter was then cloned upstream
of the gIV gene. This was accomplished by cloning
oligonucleotides, BHVL7 (SEQ ID NO:239) and BHVL8 (SEQ ID
NO:240), encoding the 3'-end of the π promoter and the 5'-
end of the gIV gene, into the 5,500 bp partial SstII-XhoI
fragment of pBHV1. The plasmid generated by this
manipulation is called pBHV3.

Extraneous 3'-noncoding sequence was then eliminated.
This was accomplished by cloning the oligonucleotides, BHVL5
(SEQ ID NO:241) and BHVL6 (SEQ ID NO:242), into the 5,200 bp
partial SmaI-PstI fragment of pBHV3. The plasmid generated
by this manipulation is called pBHV4.

Extraneous linker sequence was then eliminated. This
was accomplished by ligating the 5,200 bp PstI fragment of
pBHV4. The plasmid generated by this manipulation is called
pBHV5.

The 5'-end of the π promoter was then cloned into
pBHV5. This was accomplished by cloning the 130 bp AflII-
XhoI fragment of pPI4, containing the 5'-end of the π
promoter, into the 5,200 bp AflII-XhoI fragment of pBHV5.
The plasmid generated by this manipulation is called pBHV6.

The BHV1 gIII gene was then cloned into pBHV6. This

was accomplished by cloning the 1,600 bp Asp718-BamHI
fragment of pBHV10, containing the I3L-promoted gIII gene,
into the 5,300 bp partial BamHI-Asp718 fragment of pBHV6.
The plasmid generated by this manipulation is called pBHV11.

pBHV11 was used in in vitro recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP1083.

Immunoprecipitation analysis was performed to determine
whether vP1083 expresses authentic BHV1 gIII and gIV
glycoproteins. Vero cell monolayers were either mock
infected, infected with NYVAC or infected with vP1083 at an
m.o.i. of 10 PFU/cell. Following an hour adsorption period,
the inoculum was aspirated and the cells were overlayed with
2 mls of modified Eagle's medium (minus methionine)
containing 2% fetal bovine serum and [^{35}S]-methionine (20
μCi/ml). Cells were harvested at 7 hrs post-infection by
the addition of 1 ml 3X buffer A (3% NP-40, 30mM Tris
(pH7.4), 3mM EDTA, 0.03% Na Azide and 0.6 mg/ml PMSF) and 50
mls aprotinin, with subsequent scraping of the cell
monolayers.

Lysates were then analyzed for BHV1 gIII and gIV
expression using the BHV1 gIII-specific monoclonal antibody,
1507, and the gIV-specific monoclonal antibody, 3402
(obtained from Dr. Geoffrey Letchworth, U. of Wisconsin,
Madison, WI). This was accomplished by the following
procedure: rat anti-mouse sera was bound to protein-A
sepharose at room temperature for 4 hours. After washing
the material 5X with 1X buffer A, the protein A-sepharose
bound rat anti-mouse antibody was bound to the gIII-specific
monoclonal antibody and the gIV-specific monoclonal
antibody. The lysates, meanwhile, were precleared by
incubating normal mouse sera and the protein A-sepharose
bound rat anti-mouse antibody overnight at 4°C. After
washing this material 5X with 1X buffer A, the BHV1 gIII or
gIV-specific monoclonal antibody, rat anti-mouse, protein A-
sepharose conjugate was added to the lysate and incubated
overnight at 4°C. After washing the samples 4X with 1X
buffer A and 2X with a LiCl$_2$/urea buffer, the precipitated
proteins were dissociated from the immune complexes by the
addition of 2X Laemmli's buffer (125 mM Tris (pH6.8), 4%

SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5
min. Proteins were then fractionated on a 10% Dreyfuss gel
system (Dreyfuss et al., 1984), fixed and treated with 1M
Na-salicylate for fluorography.

The BHV1 gIII and gIV-specific monoclonal antibodies,
1507 and 3402, specifically precipitated the BHV1 gIII and
gIV glycoproteins from vP1083 infected cells, but did not
precipitate BHV1-specific proteins from mock or NYVAC
infected cells.

Generation of NYVAC/BHV1 gI and gIII recombinant. A
plasmid, pBHVgIII, containing the BHV1 gIII gene was
obtained from Rhone Merieux. The gIII gene from this
plasmid was cloned between vaccinia virus flanking arms.
This was accomplished by cloning the 1,000 bp NcoI-XhoI
fragment of pBHVgIII, containing the 5'-end of the gIII
gene, and the oligonucleotides, BHVL1 (SEQ ID NO:249) and
BHVL2 (SEQ ID NO:250), encoding the I3L promoter, into the
BamHI-XhoI site of pSD544VC. The plasmid generated by this
manipulation is called pBHV2.

The 3'-end of the gIII gene was then cloned into pBHV2.
This was accomplished by cloning the oligonucleotides,
BHVL15 (SEQ ID NO:251) and BHVL16 (SEQ ID NO:252), encoding
the 3'-end of the gIII gene, into the 4,700 bp XhoI-Asp718
fragment of pBHV2. The plasmid generated by this
manipulation is called pBHV7.

The rest of the gIII gene was then cloned into pBHV7.
This was accomplished by cloning the 500 bp partial SmaI-
XhoI fragment of pBHVgIII, containing an interior portion of
the gIII gene, into the 4,750 bp partial SmaI-XhoI fragment
of pBHV7. The plasmid generated by this manipulation is
called pBHV10.

The BHV1 gI gene was then cloned into pBHV10. This was
accomplished by cloning the 2,900 bp BglII fragment of
pBHV8, containing the H6-promoted gI gene, into the BamHI
site of pBHV10. The plasmid generated by this manipulation
is called pBHV12.

pBHV12 was used in in vitro recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP1087.

Immunoprecipitation analysis was performed to determine

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whether vP1087 expresses authentic BHV1 gI and gIII
glycoproteins. Vero cell monolayers were either mock
infected, infected with NYVAC or infected with vP1087 at an
m.o.i. of 10 PFU/cell. Following an hour adsorption period,
the inoculum was aspirated and the cells were overlayed with
2 mls of modified Eagle's medium (minus methionine)
containing 2% fetal bovine serum and [^{35}S]-methionine (20
μCi/ml). Cells were harvested at 7 hrs post-infection by
the addition of 1 ml 3X buffer A (3% NP-40, 30mM Tris
(pH7.4), 3mM EDTA, 0.03% Na Azide and 0.6 mg/ml PMSF) and 50
mls aprotinin, with subsequent scraping of the cell
monolayers.

Lysates were then analyzed for BHV1 gI and gIII
expression using the BHV1 gI-specific monoclonal antibody,
5106, and the gIII-specific monoclonal antibody, 1507
(obtained from Dr. Geoffrey Letchworth, U. of Wisconsin,
Madison, WI). This was accomplished by the following
procedure: rat anti-mouse sera was bound to protein-A
sepharose at room temperature for 4 hours. After washing
the material 5X with 1X buffer A, the protein A-sepharose
bound rat anti-mouse antibody was bound to the gI-specific
monoclonal antibody and the gIII-specific monoclonal
antibody. The lysates, meanwhile, were precleared by
incubating normal mouse sera and the protein A-sepharose
bound rat anti-mouse antibody overnight at 4°C. After
washing this material 5X with 1X buffer A, the BHV1 gI or
gIII-specific monoclonal antibody, rat anti-mouse, protein
A-sepharose conjugate was added to the lysate and incubated
overnight at 4°C. After washing the samples 4X with 1X
buffer A and 2X with a LiCl$_2$/urea buffer, the precipitated
proteins were dissociated from the immune complexes by the
addition of 2X Laemmli's buffer (125 mM Tris (pH6.8), 4%
SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5
min. Proteins were then fractionated on a 10% Dreyfuss gel
system (Dreyfuss et al., 1984), fixed and treated with 1M
Na-salicylate for fluorography.

The BHV1 gI and gIII-specific monoclonal antibodies,
5106 and 1507, specifically precipitated the BHV1 gI and
gIII glycoproteins from vP1087 infected cells, but did not

precipitate BHV1-specific proteins from mock or NYVAC
infected cells.

     Generation of NYVAC/BHV1 gI, gIII and gIV recombinant.
A plasmid, pBHVgIV, containing the BHV1 gIV gene, was
obtained from Rhone Merieux. The gIV gene from this plasmid
was cloned between vaccinia virus flanking arms. This was
accomplished by cloning the 2,000 bp PstI-XhoI fragment of
pBHVgIV, containing the gIV gene, into the PstI-XhoI site of
pSD542VCVQ. The plasmid generated by this manipulation is
called pBHV1.

     The 3'-end of the π promoter was then cloned upstream
of the gIV gene. This was accomplished by cloning the
oligonucleotides, BHVL7 and BHVL8, encoding the 3'-end of
the π promoter and the 5'-end of the gIV gene, into the
5,500 bp partial SstII-XhoI fragment of pBHV1. The plasmid
generated by this manipulation is called pBHV3.

     Extraneous 3'-noncoding sequence was then eliminated.
This was accomplished by cloning the oligonucleotides, BHVL5
(SEQ ID NO:241) and BHVL6 (SEQ ID NO:242), into the 5,200 bp
partial SmaI-PstI fragment of pBHV3. The plasmid generated
by this manipulation is called pBHV4.

     Extraneous linker sequences were then eliminated. This
was accomplished by ligating the 5,200 bp PstI fragment of
pBHV4. The plasmid generated by this manipulation is called
pBHV5.

     The 5'-end of the π promoter was then cloned into
pBHV5. This was accomplished by cloning the 130 bp AflIII-
XhoI fragment of pPI4, containing the 5'-end of the π
promoter, into the 5,200 bp AflII-XhoI fragment of pBHV5.
The plasmid generated by this manipulation is called pBHV6.

     The BHV1 gIII gene was then cloned into pBHV6. This
was accomplished by cloning the 1,600 bp Asp718-BamHI
fragment of pBHV10, containing the I3L-promoted gIII gene,
into the 5,300 bp partial BamHI-Asp718 fragment of pBHV6.
The plasmid generated by this manipulation is called pBHV11.

     The BHV1 gI gene was then cloned into pBHV11. This was
accomplished by cloning the 2,900 bp BglII fragment of
pBHV8, containing the H6-promoted gI gene, into the BglII
site of pBHV11. The plasmid generated by this manipulation

is called pBHV13.

pBHV13 was used in *in vitro* recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP1079.

Immunoprecipitation analysis was performed to determine
whether vP1079 expresses authentic BHV1 gI, gIII and gIV
glycoproteins. Vero cell monolayers were either mock
infected, infected with NYVAC or infected with vP1079 at an
m.o.i. of 10 PFU/cell. Following an hour adsorption period,
the inoculum was aspirated and the cells were overlayed with
2 mls of modified Eagle's medium (minus methionine)
containing 2% fetal bovine serum and [^{35}S]-methionine (20
μCi/ml). Cells were harvested at 7 hrs post-infection by
the addition of 1 ml 3X buffer A (3% NP-40, 30mM Tris
(pH7.4), 3mM EDTA, 0.03% Na Azide and 0.6 mg/ml PMSF) and 50
mls aprotinin, with subsequent scraping of the cell
monolayers.

Lysates were then analyzed for BHV1 gI, gIII and gIV
expression using the BHV1 gI-specific monoclonal antibody,
5106, the gIII-specific monoclonal antibody, 1507, and the
gIV-specific monoclonal antibody, 3402 (obtained from Dr.
Geoffrey Letchworth, U. of Wisconsin, Madison, WI). This
was accomplished by the following procedure: rat anti-mouse
sera was bound to protein-A sepharose at room temperature
for 4 hours. After washing the material 5X with 1X buffer
A, the protein A-sepharose bound rat anti-mouse antibody was
bound to the gI, gIII and gIV-specific monoclonal
antibodies. The lysates, meanwhile, were precleared by
incubating normal mouse sera and the protein A-sepharose
bound rat anti-mouse antibody overnight at 4°C. After
washing this material 5X with 1X buffer A, the BHV1 gI, gIII
and gIV-specific monoclonal antibody, rat anti-mouse,
protein A-sepharose conjugate was added to the lysate and
incubated overnight at 4°C. After washing the samples 4X
with 1X buffer A and 2X with a LiCl$_2$/urea buffer, the
precipitated proteins were dissociated from the immune
complexes by the addition of 2X Laemmli's buffer (125 mM
Tris (pH6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol)
and boiling for 5 min. Proteins were then fractionated on a
10% Dreyfuss gel system (Dreyfuss et al., 1984), fixed and

treated with 1M Na-salicylate for fluorography.

The BHV1 gI, gIII and gIV-specific monoclonal
antibodies, 5106, 1507 and 3402, specifically precipitated
the BHV1 gI, gIII and gIV glycoproteins from vP1079 infected
cells, but did not precipitate BHV1-specific proteins from
mock or NYVAC infected cells.

**Example 31 - EXPRESSION OF BOVINE VIRAL DIARRHEA VIRUS
                 (BVDV) GENES IN NYVAC**

Generation of NYVAC/BVDV gE1/gE2 recombinant. The BVDV
gE1 (gp48/gp25) "gene" (Osloss strain) was cloned into
pIBI25. This was accomplished by blunt-ending the 1,370 bp
EcoRI-BamHI fragment of pSP65-gE1 (obtained from Eurogentec,
Liege, Belgium; Renard et al., European Patent Application
No:86870095) with E. coli DNA polymerase I (Klenow
fragment), ligating XhoI linkers onto the ends and cloning
the resulting fragment into the XhoI site of pIBI25. The
plasmid generated by this manipulation is called pBDV1.

The initiation codon of the H6 promoter was then
aligned with the "initiation codon" of the gE1 "gene". This
was accomplished by cloning the oligonucleotides, BDVM4 (SEQ
ID NO:253) (5'-AGCTTGATATCCGTTAAGTTTGTATCGTAATGGGCAAAC
TAGAGAAAGCCCTGT-3') and BDVM5 (SEQ ID NO:254)
(5'-GGGCTTTCTCTAGTTTGCCCATTACGATACAAACTTAACGGATATCA-3'),
encoding the 3'-end of the H6 promoter and the 5'-end of the
gE1 "gene", into the 4,250 bp HindIII-BglI (partial)
fragment of pBDV1. The plasmid generated by this
manipulation is called pBDV6.

The gE1 "gene" was then cloned downstream of the
H6+ATI+HA triple promoter (Portetelle et al., Vaccine (1991)
9:194) and between HA flanking arms. This was accomplished
by cloning the 1,380 bp EcoRV-PstI (partial) fragment of
pBDV6, containing the gE1 "gene", into the 3,700 bp EcoRV-
PstI fragment of pATI25. The plasmid generated by this
manipulation is called pBDV7.

A BamHI site, necessary for future manipulations, was
then generated downstream of the BVDV sequence. This was
accomplished by cloning the oligonucleotide, BDVM6 (SEQ ID
NO:255) (5'-TCGAGGATCC-3'), into the XhoI site of pBDV7.
The plasmid generated by this manipulation is called pBDV8.

Approximately 830 bp of gE2 (gp53) sequence (Osloss
strain) was then cloned downstream of the gE1 sequence.
This was accomplished by cloning the 980 bp BglII-BamHI
fragment of p7F2 (obtained from Eurogentec, Liege, Belgium;
Renard et al., European Patent Application No:86870095),
containing the gE2 sequence, into the 5,100 bp BamHI-BglII
(partial) fragment of pBDV8. The plasmid generated by this
manipulation is called pBDV9.

The H6 promoted-gE1/gE2 sequence was then cloned
between ATI flanking arms. This was accomplished by cloning
the 2,200 bp NruI-BamHI fragment of pBDV9, containing the
gE1/gE2 sequence, into the 4,900 bp NruI-BamHI fragment of
pPGI7. This places the gE1/gE2 sequence under the
transcriptional control of the H6 promoter and into an
insertion vector. The plasmid generated by this
manipulation is called pBDV23.

Approximately 270 bp of additional gE2 sequence (Osloss
strain) was then cloned downstream of the existing BVDV
sequence. This was accomplished by cloning the 1,260 bp
BglII-BamHI fragment of pSP65E1+E2-1 (obtained from
Eurogentec, Liege, Belgium; Renard et al., European Patent
Application No:86870095), containing the gE2 sequence, into
the 6,100 bp fragment of pBDV23. The plasmid generated by
this manipulation is called pBDV24.

pBDV24 was used in *in vitro* recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP972.

Immunoprecipitation analysis was performed to determine
whether vP972 expresses authentic BVDV gE1 and gE2
glycoproteins. Vero cell monolayers were either mock
infected, infected with NYVAC or infected with vP972 at an
m.o.i. of 10 PFU/cell. Following an hour adsorption period,
the inoculum was aspirated and the cells were overlayed with
2 mls of modified Eagle's medium (minus methionine)
containing 2% fetal bovine serum and [^{35}S]-methionine (20
μCi/ml). Cells were harvested at 18 hrs post-infection by
the addition of 1 ml 3X buffer A (3% NP-40, 30mM Tris
(pH7.4), 3mM EDTA, 0.03% Na Azide and 0.6 mg/ml PMSF) and 50
mls aprotinin, with subsequent scraping of the cell
monolayers.

Lysates were then analyzed for BVDV gE1 and gE2
expression using the BVDV gp48-specific monoclonal
antibodies, NYC16 and NY12B1, and the BVDV gp53-specific
monoclonal antibody, 209D3 (obtained from Rhone Merieux,
Lyon, France). This was accomplished by the following
procedure: rat anti-mouse sera was bound to protein-A
sepharose at room temperature for 4 hours. After washing
the material 5X with 1X buffer A, protein A-sepharose bound
rat anti-mouse antibody was bound to the gE1-specific
monoclonal antibodies, NYC16 and NY12B1, and the gE2-
specific monoclonal antibody, 209D3. The lysates,
meanwhile, were precleared by incubating normal mouse sera
and the protein A-sepharose bound rat anti-mouse antibody
overnight at 4°C. After washing this material 5X with 1X
buffer A, the BVDV gE1 or gE2-specific monoclonal antibody,
rat anti-mouse, protein A-sepharose conjugate was added to
the lysate and incubated overnight at 4°C. After washing
the samples 4X with 1X buffer A and 2X with a LiCl$_2$/urea
buffer, the precipitated proteins were dissociated from the
immune complexes by the addition of 2X Laemmli's buffer (125
mM Tris (pH6.8), 4% SDS, 20% glycerol, 10% 2-
mercaptoethanol) and boiling for 5 min. Proteins were then
fractionated on a 10% Dreyfuss gel system (Dreyfuss et al.,
1984), fixed and treated with 1M Na-salicylate for
fluorography.

The BVDV gE1 or gE2-specific monoclonal antibodies
precipitated BVDV-specific glycoproteins from vP972 infected
cells, but did not precipitate BVDV-specific proteins from
NYVAC or mock infected cells.

     Generation of NYVAC/BVDV CAPSID/gE1/gE2 recombinant.
The BVDV gE1 "gene" was cloned into pIBI25. This was
accomplished by blunt-ending the 1,370 bp EcoRI-BamHI
fragment of pSP65-gE1, containing the gE1 "gene", with E.
coli DNA polymerase I (Klenow fragment), ligating XhoI
linkers onto the ends and cloning the resulting fragment
into the XhoI site of pIBI25. The plasmid generated by this
manipulation is called pBDV1.

The gE1 "gene" was then cloned between u flanking arms.
This was accomplished by cloning the 1,400 bp XhoI fragment

of pBDV1, containing the gE1 sequence, into the XhoI site of
pSD486. The plasmid generated by this manipulation is
called pBDV11.

The "initiation codon" of the gE1 "gene" was then
aligned with the initiation codon of u promoter. This was
accomplished by cloning the oligonucleotides, BDVM7 (SEQ ID
NO:256) (5'-CGATTACTATGGGCAAACTAGAGAAAGCCCTGT-3') and BDVM8
(SEQ ID NO:257) (5'-GGGCTTTCTCTAGTTTGCCCATAGTAAT-3'),
encoding the 3'-end of the u promoter and the 5'-end of the
gE1 sequence, into the 4,800 bp partial BglI-ClaI fragment
of pBDV11. The plasmid generated by this manipulation is
called pBDV12.

Part of the BVDV gE2 "gene" was then cloned into
pBDV12, downstream from the gE1 sequence. This was
accomplished by cloning the 1,000 bp BglII-BamHI fragment of
p7F2, containing the gE2 sequence, into the 4,650 bp BglII-
BamHI fragment of pBDV12. The plasmid generated by this
manipulation is called pBDV14.

The rest of the gE2 "gene" was then cloned into pBDV14.
This was accomplished by cloning the 1,260 bp BglII-BamHI
fragment of pSP65E1+E2-1, containing the gE2 "gene", into
the 4,650 bp BglII-BamHI fragment of pBDV14. The plasmid
generated by this manipulation is called pBDV17.

The capsid "gene" (Osloss strain) was then cloned into
pBDV17, upstream from the gE1 sequence. This was
accomplished in 2 steps. The first step aligned the
initiation codon of the u promoter with the "initiation
codon" of the capsid "gene". This was accomplished by
cloning the oligonucleotides, BDVL12 (SEQ ID NO:258)
(5'-CGATTACTATGGAGTTGATTACAAATGAACTTTTATACAAAACATACAAAC
AAAAACCCGCTGGAGTGGAGGAACCAGTATATAACCAAGCAGGTGACCCT-3') and
BDVL13 (SEQ ID NO:259) (5'-CTAGAGGGTCACCTGCTTGGTTATATA
CTGGTTCCTCCACTCCAGCGGGTTTTTGTTTGTATGTTTTGTATAAAAGTTCATTTGTAA
TCAACTCCATAGTAAT-3'), encoding the 3'-end of the u promoter
and the 5'-end of the capsid sequence, into the 5,200 bp
ClaI-XbaI fragment of pBDV17. The plasmid generated by this
manipulation is called pBDV25. The second step cloned the
rest of the capsid "gene" into pBDV25. This was
accomplished by cloning the 1,870 bp BstEII-BglII fragment

of pSP65C-E1-E2 (obtained from Eurogentec, Liege, Belgium; Renard et al., European Patent Application No:86870095), containing the capsic "gene", into the 4,700 bp BstEII-BglII fragment of pBDV25. The plasmid generated by this manipulation is called pBDV26.

The u-promoted capsid/gE1/gE2 sequence was then cloned between tk flanking arms. This was accomplished by cloning the 3,200 bp SnaBI-BamHI fragment of pBDV26, containing the u-promoted capsid/gE1/gE2 sequence, into the 4,000 bp SmaI-BamHI fragment of pSD542. The plasmid generated by this manipulation is called pBDV27.

pBDV27 was used in in vitro recombination experiments with vP866 (NYVAC) as the rescuing virus to yield vP1017.

Immunoprecipitation experiments with vP1017 infected cells were performed as described above for the expression of vP972. No BVDV-specific proteins were precipitated from mock infected or NYVAC infected Vero cells. BVDV-specific proteins were precipitated, however, from lysates of vP1017.

Generation of NYVAC/BVDV gE2 recombinant. The BVDV gE1 "gene" was cloned into pIBI25. This was accomplished by blunt-ending the 1,370 bp EcoRI-BamHI fragment of pSP65-gE1, containing the gE1 "gene", with E. coli DNA polymerase I (Klenow fragment), ligating XhoI linkers onto the ends and cloning the resulting fragment into the XhoI site of pIBI25. The plasmid generated by this manipulation is called pBDV1.

The initiation codon of the H6 promoter was then aligned with the putative "initiation codon" of the gE1 "gene". This was accomplished by cloning the oligonucleotides, BDVM4 (SEQ ID NO:253) and BDVM5 (SEQ ID NO:254), encoding the 3'-end of the H6 promoter and the 5'-end of the gE1 "gene", into the 4,250 bp HindIII-BglI (partial) fragment of pBDV1. The plasmid generated by this manipulation is called pBDV6.

The gE1 "gene" was then cloned downstream of the H6+ATI+HA triple promoter and between HA flanking arms. This was accomplished by cloning the 1,380 bp EcoRV-PstI (partial) fragment of pBDV6, containing the gE1 "gene", into the 3,700 bp EcoRV-PstI fragment of pATI25. The plasmid generated by this manipulation is called pBDV7.

A BamHI site, necessary for future manipulations, was then generated downstream of the BVDV sequence. This was accomplished by cloning the oligonucleotide, BDVM6 (SEQ ID NO:255), into the XhoI site of pBDV7. The plasmid generated by this manipulation is called pBDV8.

Approximately 830 bp of BVDV gE2 sequence was then cloned downstream of the gE1 "gene". This was accomplished by cloning the 980 bp BglII-BamHI fragment of p7F2, containing the gE2 sequence, into the 5,100 bp BamHI-BglII (partial) fragment of pBDV8. The plasmid generated by this manipulation is called pBDV9.

The gE1/gE2 sequence was then cloned between ATI flanking arms. This was accomplished by cloning the 2,200 bp NruI-BamHI fragment of pBDV9, containing the H6-promoted gE1/gE2 "genes", into the 4,900 bp NruI-BamHI fragment of pPGI7. The plasmid generated by this manipulation is called pBDV23.

Approximately 270 bp of additional gE2 sequence was then cloned downstream of the existing BVDV sequence. This was accomplished by cloning the 1,260 bp BglII-BamHI fragment of pSP65E1+E2-1, containing the additional gE2 sequence, into the 6,100 bp BamHI-BglII (partial) fragment of pBDV23. The plasmid generated by this manipulation is called pBDV24.

The gE1 sequence was then deleted from BDV24. This was accomplished by cloning a 130 bp NruI-PstI PCR fragment, containing the 3'-end of the H6 promoter and the 5'-end of the gE2 "gene", into the 5,900 bp NruI-PstI fragment of pBDV24. This PCR fragment was generated from the plasmid, pBDV17, with the oligonucleotides, BDVP14 (SEQ ID NO:260) (5'-TTTCGCGATATCCGTTAAGTTTGTATCGTAATGCTCCCAGTTTGCAAACCC-3') and BDVP15 (SEQ ID NO:261) (5'-TCTCCACCTTTACACCACACT-3'). The plasmid generated by this manipulation is called pBDV28.

Sequence analysis revealed that the H6 promoter in pBDV28 contains a 2 bp insertion. To correct this error, the 130 bp NruI-PstI fragment of pBDV28, containing the 3'-end of the H6 promoter and the 5'-end of the gE2 "gene", was cloned into the 5,900 bp NruI-PstI fragment of pBDV24. The plasmid generated by this manipulation is called pBDV29.

pBDV29 was used in in vitro recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP1097.

Immunoprecipitation experiments with vP1097 infected
cells are performed as described above to yield BVDV
proteins from cells or lysates.

Example 32 - **CLONING AND EXPRESSION OF HUMAN
                CYTOMEGALOVIRUS (HCMV) GLYCOPROTEIN ANTIGENS
                IN POXVIRUS VECTORS**

Cloning of the HCMV gB gene into the NYVAC donor
plasmid, pSD542. The 4800 bp HindIII-BamHI fragment of the
HindIII D fragment of the HCMV DNA was cloned into the 2800
bp HindIII-BamHI fragment of the plasmid pIBI24. By in vitro
mutagenesis (Kunkel, 1985; Russel et al., 1986) using the
oligonucleotides CMVM5 (SEQ ID NO:262) (5'-GCCTCATCGCTGCT
GGATATCCGTTAAGTTTGTATCGTAATGGAATCCAGGATCTG-3') and CMVM3
(SEQ ID NO:263) (5'-GACAGATTGTGATTTTTATAAGCATCGTAAGC
TGTCA-3'), the gB gene was modified to be expressed under
the control of the vaccinia H6 promoter (Taylor et al.,
1988a,b; Perkus et al., 1989). The plasmid containing the
modified gB was designated 24CMVgB(5+3).

The 2900 bp EcoRV-BamHI fragment of 24CMVgB(5+3) was
cloned into the 3100 bp EcoRV-BglII fragment of pSP131. This
cloning step put the gB gene under the control of the H6
promoter. The resulting plasmid was designated SP131gB.

To modify the restriction sites flanking the H6
promoted gB in SP131gB the following steps were performed.
Plasmid pMP22BHP contains a subclone of the HindIII F
fragment of vaccinia (WR strain) containing a portion of the
HBV sAg in a polylinker region at the BamHI site. pMP22BHP
was digested within the polylinker with HindIII and ligated
to a HindIII fragment from SP131CMVgB (containing the H6
promoted gB gene) generating plasmid SAg22CMVgB. SAg22CMVgB
was digested with BamHI and partially digested with HindIII
and ligated to a polylinker derived from pIBI24 by BamHI and
HindIII digestion creating plasmid 22CMVgB which contains
the H6 promoted gB gene without the HBV sAg.

Plasmid pSD542 (a NYVAC TK locus donor plasmid) was
derived from plasmid pSD460 (Tartaglia et al., 1992) by
forming vector plasmid pSD513 as described above in Example
7. The polylinker region in pSD513 was modified by cutting

with PstI/BamHI and ligating to annealed synthetic
oligonucleotides MPSYN288 (SEQ ID NO:264)
(5' GGTCGACGGATCCT 3') and MPSYN289 (SEQ ID NO:265)
(5' GATCAGGATCCGTCGACCTGCA 3') resulting in plasmid pSD542.

    22CMVgB was digested with BamHI and NsiI to generate a
fragment containing the H6 promoter and part of the gB gene,
and with NsiI and PstI to generate a fragment containing the
remainder of the gB gene. These two fragments were ligated
to pSD542 that had been digested with BamHI and PstI within
its' polylinker creating the NYVAC donor plasmid 542CMVgB.

    Cloning of the HCMV gB size into the ALVAC donor
plasmid CP3LVQH6. An 8.5kb canarypox BglII fragment was
cloned in the BamHI site of pBS-SK plasmid vector to form
pWW5. Nucleotide sequence analysis revealed a reading frame
designated C3. In order to construct a donor plasmid for
insertion of foreign genes into the C3 locus with the
complete excision of the C3 open reading frame, PCR primers
were used to amplify the 5' and 3' sequences relative to C3.
Primers for the 5' sequence were RG277 (SEQ ID NO:177) and
RG278 (SEQ ID NO:178).

    Primers for the 3' sequences were RG279 (SEQ ID NO:179)
and RG280 (SEQ ID NO:180). The primers were designed to
include a multiple cloning site flanked by vaccinia
transcriptional and translational termination signals. Also
included at the 5'-end and 3'-end of the left arm and right
arm were appropriate restriction sites (Asp718 and EcoRI for
left arm and EcoRI and SacI for right arm) which enabled the
two arms to ligate into Asp718/SacI digested pBS-SK plasmid
vector. The resultant plasmid was designated as pC3I.

    A 908 bp fragment of canarypox DNA, immediately
upstream of the C3 locus was obtained by digestion of
plasmid pWW5 with NsiI and SspI. A 604 bp fragment of
canarypox and DNA was derived by PCR (Engelke et al., 1988)
using plasmid pWW5 as template and oligonucleotides CP16
(SEQ ID NO:266) (5'-TCCGGTACCGCGGCCGCAGATATTTGTTAGCTTC
TGC-3') and CP17 (SEQ ID NO:267) (5'-TCGCTCGAGTAG
GATACCTACCTACTACCTACG-3'). The 604 bp fragment was digested
with Asp718 and XhoI (sites present at the 5' ends of
oligonucleotides CP16 and CP17, respectively) and cloned

into Asp718-XhoI digested and alkaline phosphatase treated
IBI25 (International Biotechnologies, Inc., New Haven, CT)
generating plasmid SPC3LA. SPC3LA was digested within IBI25
with EcoRV and within canarypox DNA with NsiI and ligated to
the 908 bp NsiI-SspI fragment generating SPCPLAX which
contains 1444 bp of canarypox DNA upstream of the C3 locus.

A 2178 bp BglII-StyI fragment of canarypox DNA was
isolated from plasmids pXX4 (which contains a 6.5 kb NsiI
fragment of canarypox DNA cloned into the PstI site of pBS-
SK. A 279 bp fragment of canarypox DNA was isolated by PCR
(Engelke et al., 1988) using plasmid pXX4 as template and
oligonucleotides CP19 (SEQ ID NO:268) (5'-TCGCTCGAGCTTTC
TTGACAATAACATAG-3') and CP20 (SEQ ID NO:269) (5'-TAGGAGC
TCTTTATACTACTGGGTTACAAC-3'). The 279 bp fragment was
digested with XhoI and SacI (sites present at the 5' ends of
oligonucleotides CP19 and CP20, respectively) and cloned
into SacI-XhoI digested and alkaline phosphatase treated
IBI25 generating plasmid SPC3RA.

To add additional unique sites to the polylinker, pC3I
was digested within the polylinker region with EcoRI and
ClaI, treated with alkaline phosphatase and ligated to
kinased and annealed oligonucleotides CP12 (SEQ ID NO:272)
and CP13 (SEQ ID NO:273) (containing an EcoRI sticky end,
XhoI site, BamHI site and a sticky end compatible with ClaI)
generating plasmid SPCP3S.
CP12 (SEQ ID NO:272)   5'-AATTCCTCGAGGGATCC -3'
CP13 (SEQ ID NO:273)   3'-   GGAGCTCCCTAGGGC-5'
                          EcoRI   XhoI BamHI

SPCP3S was digested within the canarypox sequences
downstream of the C3 locus with StyI and SacI (pBS-SK) and
ligated to a 261 bp BglII-SacI fragment from SPC3RA and the
2178 bp BglII-StyI fragment from pXX4 generating plasmid
CPRAL containing 2572 bp of canarypox DNA downstream of the
C3 locus. SPCP3S was digested within the canarypox sequences
upstream of the C3 locus with Asp718 (in pBS-SK) and AccI
and ligated to a 1436 bp Asp718-AccI fragment from SPCPLAX
generating plasmid CPLAL containing 1457 bp of canarypox DNA
upstream of the C3 locus. CPLAL was digested within the
canarypox sequences downstream of the C3 locus with StyI and
SacI (in pBS-SK) and ligated to a 2438 bp StyI-SacI fragment

from CPRAL generating plasmid CP3L containing 1457 bp of
canarypox DNA upstream of the C3 locus, stop codons in six
reading frames, early transcription termination signal, a
polylinker region, early transcription termination signal,
stop codons in six reading frames, and 2572 bp of canarypox
DNA downstream of the C3 locus. The resulting plasmid was
designated SPCP3L.

The early/late H6 vaccinia virus promoter (Guo et al.,
1989; Perkus et al., 1989) was derived by PCR (Engelke et
al., 1988) using pRW838 as template and oligonucleotides
CP21 (SEQ ID NO:270) (5'-TCGGGATCCGGGTTAATTAAT
TAGTTATTAGACAAGGTG-3') and CP22 (SEQ ID NO:271)
(5'-TAGGAATTCCTCGAGTACGATACAAACTTAAGCGGATATCG-3'). The PCR
product was digested with BamHI and EcoRI (sites present at
the 5' ends of oligonucleotides CP21 and CP22, respectively)
and ligated to CP3L that was digested with BamHI and EcoRI
in the polylinker generating plasmid VQH6CP3L.

ALVAC donor plasmid VQH6CP3L was digested within the
polylinker with XhoI and within the H6 promoter with NruI
and ligated to a NruI/HindIII fragment from 22CMgB
containing part of the H6 promoter and gB gene and a
polylinker derived from pIBI24 by XhoI and HindIII digestion
generating the ALVAC donor plasmid CP3LCMVgB.

**Example 33 - CONSTRUCTION OF RECOMBINANT VIRUSES:
                 CYTOMEGALOVIRUS**

The CMV (cytomegalovirus) gB gene was inserted into the
TK site of NYVAC. The recombinant virus was designated
vP1001. The CMV gB gene was inserted into the C3 site of
ALVAC. The recombinant was designated vCP139.

**Example 34 - IMMUNOFLUORESCENCE OF CMV GB PROTEIN IN
                 RECOMBINANT VIRUS INFECTED CELLS**

Immunofluorescence studies were performed as described
previously (Taylor et al., 1990) using guinea pig polyclonal
serum followed by fluorescein isothiocyanate goat anti-
guinea pig. Cells infected with vP1001 showed gB expressed
on the plasma membrane. Weak internal expression was
detected within cells infected with vCP139.

Example 35 - **IMMUNOPRECIPITATION OF CMV GB IN RECOMBINANT INFECTED CELLS**

Immunoprecipitation experiments were performed as described previously (Taylor et al., 1990). The CMV gB glycoprotein produced in CMV infected cells has a molecular weight of 55 kDa with a precursor form of 130 kDa (Gretch et al., 1988). Cells infected with vP1001 and vCP139 produce two CMV gB coded proteins of approximately 116 kDa and 55 kDa.

Example 36 - **NEUTRALIZING ANTIBODIES**

Following immunization of CBA mice with vP1001 (NYVAC-HCMV gB), neutralizing antibody titers of the sera of inoculated mice were assessed (Gonczol et al., 1986). Antibodies capable of neutralizing human cytomegalovirus were detected in the sera of mice 14-21 days later (geometric mean titers of 1:16) and between 28 and 60 days post-immunization (gmt=1:26). Immunization of CBA mice with ALVAC-HCMV gB generated HCMV neutralizing antibody titers of 1:64 gmt (14-21 days pi, 1:91 gmt between 21 and 28 days pi), and 1:111 between 28 and 60 days pi. Thus, immunization of mice with vaccinia virus or canarypox virus recombinants expressing HCMV gB elicited antibodies able to neutralize the infectivity of HCMV.

Example 37 - **CELL MEDIATED IMMUNITY**

Besides HCMV neutralizing antibody titers, vCP139 is also capable of eliciting cytotoxic T lymphocytes capable of killing murine L929 cells infected with a recombinant vaccinia virus expressing HCMV gB (vaccinia WR-gB). CBA mice were immunized intraperitoneally with 2.5×10^8 pfu of vCP139. Sixteen to 30 days later, spleen cell suspensions of the mice were re-stimulated in vitro by co-incubation with syngeneic spleen cells previously infected with vP1001 at a ratio of 2:1. After 5 days, the spleen cells were counted and, using the ^{51}Cr-release assay (Zinkernagel et al., 1984), assessed for cytotoxicity against uninfected L929 cells or L929 cells infected with adenovirus Ad5d1E3, recombinant adenovirus expressing HCMV gB (Ad-gB), vaccinia virus, and recombinant vaccinia virus expressing HCMV gB. Only background levels of reactivity were measured against

the uninfected targets as well as the targets infected with
Ad5dlE3. In contrast, the in vitro stimulated spleen cells
readily killed L929 cells infected with Ad-gB expressing
HCMV gB. Although some lytic reactivity was observed against
targets infected with the vaccinia virus vector, much higher
cytolysis was measured against targets infected with the
recombinant vaccinia virus expressing gB. This clearly
demonstrated that cytotoxic T lymphocytes specific for
epitopes located within HCMV gB were generated by
inoculation with the recombinant canarypox virus expressing
HCMV gB (vCP139).

Example 38 - **NYVAC AND ALVAC DONOR PLASMID CONSTRUCTION:**
                  CANINE PARVOVIRUS

     In order to generate poxvirus recombinants expressing
the canine parvovirus VP2 capsid gene, donor plasmids were
constructed in which the VP2 gene was amplified from the
genome of the CPV-d isolate (CPV-2 antigenic type), coupled
to the vaccinia H6 promoter (Perkus et al., 1989) and
inserted into NYVAC or ALVAC insertion vectors. The NYVAC
insertion site is the deorfed ATI locus while the ALVAC
insertion site is the deorfed C3 locus.

          The VP2 gene sequences were obtained by PCR from
the plasmid pBI265(1). This plasmid, obtained from Dr.
Colin R. Parrish, James A. Baker Institute, Cornell
University, Ithaca, NY, contains the genome of the CPV-d
isolate (Cornell 790320)(antigenic type CPV-2). The DNA
sequence of the VP2 gene from this isolate has been
published (Parrish et al., 1988).

     Using pBI265(1) as template and synthetic
oligonucleotides RG451 (SEQ ID NO:274) (5'-TCGGGT
ACCTCGCGATATCCGTTAAGTTTGTATCGTAATGAGTGATGGAGCAGT-3') and
RG452 (SEQ ID NO:275) (5'-TAGGAATTCCTCGAGTTAA
TATAATTTTCTAGGTGC-3') as primers, the complete VP2 open
reading frame (ORF) was amplified by PCR. The purified DNA
fragment was cut with Asp718 and EcoRI and cloned into the
Asp718 and EcoRI sites in pBluescript SK+, resulting in
pDT4. The VP2 gene was confirmed by DNA sequence analysis.

     The VP2 gene contains two TTTTTNT sequences within the
ORF which could function as early transcriptional stop

signals (Yuen et al., 1987). To eliminate these signals,
PCR site-directed mutagenesis was used to change the
nucleotide sequence while retaining the correct amino acid
sequence. A 250 bp fragment was amplified from pBI265(1)
using synthetic oligonucleotides RG453 (SEQ ID NO:276)
(5'-ATCAGATCTGAGACATTGGGTTTCTATCCATG-3') and RG454 (SEQ ID
NO:277) (5'-TTAGTCTACATGGTTTACAATCAAAGAAGAATGTTCCTG-3').
The purified fragment was digested with BglII and AccI, and
used to replace the BglII/AccI VP2 fragment in pDT4. The
resulting plasmid, pED3, contains the modified VP2 gene in
which the TTTTTNT sequences have been changed to TTTCTAT and
TTCTTCT.

     A NYVAC donor plasmid containing the CPV VP2 capsid
gene was constructed as follows. The modified VP2 gene was
excised from pED3 with NruI and XhoI and cloned into
pMPATIH6HSVTK cut with NruI/XhoI. pMPATIH6HSVTK is a
derivative of pSD552 (described elsewhere in this
disclosure) in which an expression cassette containing the
coding sequences for the HSV-1 thymidine kinase gene under
control of the H6 promoter is inserted between the HpaI and
XhoI sites in the polylinker region. Cutting this plasmid
with NruI and XhoI excises the tk gene, but retains the
5'end of the H6 promoter. Insertion of the modified VP2
gene into this vector as described above generates pATI-VP2.
This NYVAC donor plasmid contains the H6 promoted VP2 gene
flanked by the ATI insertion arms.

     An ALVAC donor plasmid containing the CPV VP2 capsid
gene was constructed as follows. The modified VP2 gene was
excised from pED3 with NruI and XhoI and the purified
fragment was cloned into pVQH6CP3L (plasmid described in
Flavivirus section) cut with NruI and XhoI. The resulting
plasmid, pC3-VP2, contains the H6 promoted VP2 gene flanked
by the C3 insertion arms.

**Example 39 - GENERATION OF NYVAC AND ALVAC RECOMBINANTS:
                CANINE PARVOVIRUS (CPV)**

     The donor plasmid pATI-VP2 was used in *in vitro*
recombination experiments in VERO cells with NYVAC (vP866)
and NYVAC-RG (vP879) as rescue viruses to yield vP998 and
vP999 respectively (Tartaglia et al., 1992). Recombinant

viruses were identified by in situ hybridization procedures
(Piccini et al., 1987) using a radiolabelled VP2 specific
DNA probe. Recombinant plaques were purified by three
rounds of plaque purification and amplified for further
analysis.

The donor plasmid pC3-VP2 was used in in vitro
recombination experiments in CEF cells with ALVAC (CPpp) and
ALVAC-RG (vCP65A) as rescue viruses to yield vCP123 and
vCP136 respectively (Taylor et al., 1992). Recombinant
viruses were identified by in situ hybridization procedures
(Piccini et al., 1987) using a radiolabelled VP2 specific
probe (positive signal) and C3 ORF specific probe (negative
signal). Recombinant plaques were purified by three rounds
of plaque purification and amplified for further analysis.

**Example 40 - EXPRESSION ANALYSIS OF THE NYVAC- AND ALVAC-
                BASED CPV VP2 RECOMBINANTS**

All the recombinants containing the CPV VP2 gene were
tested for expression by immunofluorescence as previously
described (Taylor et al., 1990) using monoclonal antibodies
specific for VP2 epitopes or polyclonal CPV dog serum. All
sera were obtained from Dr. Colin R. Parrish, James A. Baker
Institute, Cornell University, Ithaca, NY. The NYVAC-based
recombinants were tested on VERO cells while the ALVAC-based
recombinants were tested on CEF cells. Recombinants vP998,
vP999, vCP123, and vCP136 all displayed internal
fluorescence, with localization in the nucleus. No surface
fluorescence was detected. In addition, the two
recombinants containing the rabies G gene (vP999 and vCP136)
were screened with monoclonal antibodies specific for rabies
G epitopes. Both displayed strong fluorescence on the
surface of the cell.

To further characterize expression of an authenic VP2
gene product in the above recombinants, immunoprecipitation
analysis was done using the same antisera (Taylor et al.,
1990). The NYVAC-based recombinants were tested on VERO
cells while the ALVAC-based recombinants were tested on CEF
cells. In all recombinants (vP998, vP999, vCP123, and
vCP136) the antisera precipitated a protein of 65 kDa, which
is consistent with the size of the native VP2 gene. No

protein of this size was detected from cell lysates or from
either parental virus (NYVAC or ALVAC).

**Example 41 - INSERTION OF EPSTEIN BARR VIRUS (EBV) GENES
                 INTO ALVAC**

     Construction of donor plasmid EBV Triple.2. Plasmid
EBV Triple.1 (Example 11) contains expression cassettes for
EBV genes gH, gB and gp340 all inserted into a vaccinia TK
locus insertion plasmid. Plasmid EBV Triple.1 was digested
with SmaI/BamHI and a 0.3 kb fragment containing the 42kDa
Entomopox virus promoter and the 5' end of the EBV gH gene
was isolated. EBV Triple.1 plasmid was also digested with
BamHI and a 7.3 kb fragment containing the 3' end of the EBV
gH gene, the EBV gB expression cassette, and the EBV gp340
expression cassette was isolated. These two fragments were
then ligated into the ALVAC C5 locus insertion plasmid
pNVQC5LSP7 (described herein, see Tetanus example) which had
been cut with SmaI/BamHI. The resulting plasmid was
designated EBV Triple.2.

     Insertion of EBV genes into ALVAC. Plasmid EBV
Triple.2, containing expression cassettes for the three EBV
genes, gH, gB and gp340, in the C5 insertion locus, was used
as donor plasmid for recombination with ALVAC, generating
ALVAC recombinant vCP167.

     Expression of EBV proteins by vP944 and vCP157.
Metabolically labelled lysates from cells infected with
ALVAC recombinant vCP167 and vP944, the NYVAC-based
recombinant containing the same three genes (Example 11),
were subjected to immunoprecipitation using human polyclonal
serum to EBV as well as mouse monoclonal antibodies to EBV
gB and gp340. Precipitates were analyzed by SDS-
polyacrylamide gel electrophoresis followed by
radioautography. Proteins of the correct molecular weights
and specificities for EBV gB, gH and gp340 were observed for
both NYVAC-based recombinant vP944 and ALVAC-based
recombinant vCP167.

**Example 42 - CONSTRUCTION OF AN EXPRESSION CASSETTE FOR
                 INSERTION ON EQUINE INFLUENZA HA
                 (A1/PRAGUE/56) INTO NYVAC AND ALVAC**

     Purified EIV (A1/Prague/56) genomic RNA was provided by
Rhone-Merieux (Lyon, France). EIV-specific cDNA was

prepared as described by Gubler and Hoffman (1983).
Oligonucleotide EIVSIP (SEQ ID NO:278) (5'-ATCATCCT
GCAGAGCAAAAGCAGG-3') was used to synthesize first strand
cDNA. This oligonucleotide (SEQ ID NO:278) is complementary
to the 3'-end of each genomic RNA segment. As per Gubler
and Hoffman (1983), the cDNA is dG-tailed and inserted into
pMG5 digested with EcoRV and dC-tailed. Insertion of the
cDNA in this manner in pMG5 creates a BamHI site on both
plasmid/cDNA sequence borders.

Five hundred colonies from this EIV cDNA library were
transferred in duplicate to LB-agar plates containing
ampicillin (50 μg/ml). The colonies were transferred to
nitrocellulose for hybridization with a radiolabeled EIV HA-
specific probe. This probe was derived by using
radiolabeled first strand cDNA synthesized with
oligonucleotide EIVSIP (SEQ ID NO:278) and purified HA
genomic RNA as template. The HA genomic segment was
purified from a 1.2% low melting point agarose gel (Bethesda
Research Laboratories, Gaithersburg, MD). Total genomic RNA
was fractionated in this gel system run at 2 volts/cm in 1X
TBE. HA RNA was recovered by excising the HA band and
melting the agarose at 75°C followed by two cycles of phenol
extraction, one ether extraction, and ETOH precipitation.

Colony hybridization was performed according to
standard procedures (Maniatis et al., 1991) and a cDNA clone
containing a 1.4 kb HA cDNA insert was identified. The
clone was confirmed to be HA-specific by Northern blot
analysis versus genomic RNA and nucleotide sequence
analysis. This 1.4 kb fragment was used to generate a
radiolabeled HA-specific DNA probe for subsequent cDNA
library screenings.

Using the probe, other HA-specific cDNA clones were
identified. The largest were of 1.0 kb, 1.2 kb, and 1.4 kb
and they were designated as pEIVAIPHA-1, -10, and -8,
respectively. Collectively, these clones contain an entire
EIV HA coding sequence as determined by nucleotide sequence
analysis. The entire sequence of the EIV HA (A1/Prague/56)
determined from these analyses is provided in FIG. 23 (SEQ
ID NO:279).

A full-length cDNA clone of the EIV HA was next
generated by splicing segments from different cDNA clones.
The 5'-most 1200 bp of the HA coding sequence was derived
from pEIVAIPHA-8 by PCR using this plasmid as template and
oligonucleotides EIVSIP (SEQ ID NO:278) and EIVAIP7A (SEQ ID
NO:280) (5'-GTTGGTTTTTTCTATTAG-3'). This 1200 bp fragment
was digested with PstI creating PstI cohesive ends at both
the 5' and 3' termini. The 3'-most 600 bp of the HA coding
sequence was derived from pEIVAIPHA-10 by digestion with
BamHI and PstI. These two fragments were inserted into pBS-
SK (Stratagene, La Jolla, CA) digested with PstI and BamHI.
The plasmid generated containing the entire EIV HA
(A1/Prague/56) coding sequence was designated as
pBSEIVAIPHA.

The EIV HA coding sequence (ATG to TAA) was derived by
PCR from pBSEIVAIPHA using oligonucleotides EIVAIPHA5P (SEQ
ID NO:281) (5'-CGATATCCGTTAAGTTTGTATCGTAATGAA
GACTCAAATTCTAATATTAGCC-3') and EIVAIPHA3P (SEQ ID NO:282)
(5'-ATCATCGGATCCATAAAAATTATATACAAATAGTGCACCG-3'). The
oligonucleotide EIVAIPHA5P (SEQ ID NO:281) provides the 3'-
most 26 bp (from NruI site) of the vaccinia virus H6
promoter (Goebel et al., 1990a,b). The 1.7 kb PCR-derived
fragment was inserted into NruI digested pCPCV1 to yield
pC3EIVAIPHA. pCPCV1 is an insertion vector which contains
the H6 promoter. Insertion of the 1.7 kb blunt-ended
fragment in the proper orientation places the EIV HA 3' to
the H6 promoter. The plasmid pCPCV1 was derived as follows.
Plasmid pFeLV1A, which contains a 2.4 kb fragment containing
the FeLV env gene (Guilhot et al., 1987) in the PstI site of
pTP15 (Guo et al., 1989) was digested with PstI to excise
the FeLV sequences and religated to yield plasmid pFeLVF4.
The vaccinia virus H6 promoter element followed by a
polylinker region were liberated from pFeLVF4 by digestion
with KpnI and HpaI. The 150 bp fragment was blunt-ended
using T4 DNA polymerase and inserted into pRW764.2, a
plasmid containing a 3.3kb PvuII genomic fragment of
canarypox DNA. pRW764.2 was linearized with EcoRI, which
recognizes a unique EcoRI site within the canarypox
sequences, and blunt-ended using the Klenow fragment of the

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E. coli DNA polymerase. The resultant plasmid was
designated as pCPCV1. This plasmid contains the vaccinia
virus H6 promoter followed by a polylinker region and
flanked by canarypoxvirus homologous sequences.

Example 43   **-**   **CONSTRUCTION OF AN EXPRESSION CASSETTE FOR
INSERTION OF EIV HA (A2/FONTAINEBLEAU/79)
INTO NYVAC AND ALVAC**

    Purified EIV (A2/Fontainebleau/79) genomic RNA was
provided by Rhone-Merieux (Lyon, France). EIV-specific cDNA
was prepared as described by Gubler and Hoffman (1983) and
as described for the EIV (A1/Prague/56) cDNA preparation.
The oligonucleotide EIVSIP (SEQ ID NO:278) was used for
first-strand cDNA synthesis.

    To screen bacterial colonies containing full-length
cDNA clones of the HA gene, eight pools of transformed
colonies were amplified in 500 ml cultures and plasmid DNA
preparations obtained by standard procedures (Sambrook et
al., 1989) Total plasmid DNA was used as template in
standard PCR reactions with oligonucleotides EIVSIP (SEQ ID
NO:278) and EIVS2H (SEQ ID NO:283) (5'-ATCATCAAGCTTAGTAGAAA
CAAGG-3'). Such a reaction would potentially amplify only
full-length cDNA sequences of all eight EIV genomic
segments, since these primers were complementary to
conserved sequences at the 5' and 3' ends of these eight
segments.

    Plasmid preparation, pPEIVA2F-5, as template generated
a 1.8 kb PCR-derived fragment consistent with the size of a
full-length HA-specific fragment. This PCR-derived fragment
was re-amplified by PCR for use as a probe against the
remainder of the cDNA library. Using this probe, clones
pEIVA2FHA-7 and -8 were identified and the cDNA insert
analyzed by nucleotide sequence analysis using custom
synthesized oligonucleotides (Goebel et al., 1990a).

    Nucleotide sequence analysis demonstrated that clones
#7 and #8 represented the 3'-most 1200 bp of the EIV
(A2/Fontainebleau/79) HA coding sequence (FIG. 24) (SEQ ID
NO:284).

    The 1200 bp EIV sequence was amplified from clone #7 by
PCR using oligonucleotides A2F3P (SEQ ID NO:285)

(5'-ATCATCACTAGTATAAAAATCAAATGCAAATGTTGCATCTGATGTTGCC-3')
and A2FBAM2 (SEQ ID NO:286) (5'-ATCATCGGATCCATCACCCG
AGCACAAACAATGAGCAG-3'). The 5'-end of this 1200 bp fragment
after digestion with BamHI corresponds to nucleotide 617 of
the complete EIV (A2/Fontainebleau/79) HA coding sequence in
FIG. 24 (SEQ ID NO:284). This fragment was also digested
with SpeI which was engineered 3' to the coding sequence
using oligonucleotide A2F3P (SEQ ID NO:285). This 1200 bp
fragment was to be co-inserted into SmaI/SpeI digested pBS-
SK (Stratagene, La Jolla, CA) with a fragment containing the
5'-most 616 bp (defined below). However, screening of
potential transformants demonstrated that only the 1200 bp
fragment was inserted. Numerous clones were chosen for
nucleotide sequence analysis.

     After nucleotide sequence analysis of numerous clones,
pBSEIVA2FHA-19 was chosen for further manipulation. This
clone contained errors near the BamHI site at nucleotide 617
(FIG. 24) (SEQ ID NO:284) and at nucleotide 1570 (FIG. 24)
(SEQ ID NO:284). To correct these errors, the following
manipulations were made. Plasmid pBSEIVA2FHA-19 was
digested with BamHI and SphI and the excised 900 bp fragment
was isolated. This fragment was co-inserted into pBS-SK
digested with SpeI/BamHI with a 250 bp SphI/SpeI fragment
encompassing the 3'-most region of the HA coding sequence.
This 250 bp PCR fragment was derived using clone #7 (above)
as template and oligonucleotides A2F3P (SEQ ID NO:285) and
A2F6 (SEQ ID NO:287) (5'-TTGACTTAACAGATGCAG-3'). The
resultant plasmid was designated as pEIVH33P.

     The 5'-most 616 bp of the HA coding sequence for the
EIV HA was generated in the following manner. First, first-
stand cDNA was generated as above. This first-strand cDNA
preparation was then used as template to amplify these
sequences by PCR using oligonucleotides A2F5P (SEQ ID
NO:288) (5'-ATGAAGACAACCATTATTTTG-3') and A2FBAM1 (SEQ ID
NO:289) (5'-TGTTGAGACTGTTACTCG-3'). This fragment was
inserted into HincII digested pBS-SK (Stratagene, La Jolla,
CA) and the resultant plasmid called pEIVH35P.

     The vaccinia virus H6 promoter sequence (Goebel et al.,
1990a,b) and the 5'-most region of the HA coding sequence

were amplified and fused in the following manner. The H6
sequences were derived from a pBS-based plasmid containing
the HIV-1 (IIIB) env gene linked precisely to the H6
promoter called pBSH6IIIBE. These sequences were amplified
by PCR using oligonucleotides H65PH (SEQ ID NO:164)
(5'-ATCATCAAGCTTGATTCTTTATTCTATAC-3') and H63P (SEQ ID
NO:291) (5'-TACGATACAAACTTAACGG-3'). The 120 bp H6 fragment
was used as template with oligonucleotides H65PH (SEQ ID
NO:164) and EIV5PACC (SEQ ID NO:292) (5'-GGTTGGGTTTTGAC
TGTAGACCCAATGGGTCAGTAGTATCAAAATAATGGTTGTCTTCATTACGATACAAACTT
AACGG-3') to yield a 161 bp fragment containing the H6
promoter and the initial 41 bp of the HA coding sequence to
the AccI restriction site. This fragment was digested with
HindIII and AccI and co-inserted into HindIII/BamHI digested
pBS-SK with the 550 bp AccI/BamHI fragment from pEIVH35P.
The resultant plasmid was designated as pH6EIVH35P.

     The entire HA expression cassette was derived by co-
insertion of the 710 bp HindIII/BamHI fragment from
pH6EIVH35P and the 1200 bp BamHI/SpeI fragment from pEIVH33P
into pBS-SK digested with HindIII and SpeI. The derived
plasmid was designated as pBSA2FHAB.

     To correct the base change noted above near the BamHI
site at nucleotide position 617, the Mandecki procedure
(Mandecki, 1986) was employed. pBSA2FHAB was linearized
with BamHI and the mutagenesis procedure performed using
oligonucleotide A2F7 (SEQ ID NO:293) (5'-CAATTTCGATAAAC
TATACATCTGGGGCATCCATCACCCGAGCACAAACAATGAGCAGACAAAATTG-3').
The plasmid containing the corrected version of the HA was
designated pBSA2FHA.

**Example 44 - CONSTRUCTION OF THE INSERTION PLASMIDS
                pEIVC5L AND pEIVHAVQVV USED TO GENERATE
                vCP128 and vP961, RESPECTIVELY**

     Plasmid pC3EIVAIPHA was digested with NruI and HindIII
to excise the 1.7kb fragment containing the 3'-most 26 bp of
the H6 promoter and the entire EIV (A1/Prague/56) HA coding
sequence. Following blunt-ending with Klenow, this fragment
was inserted into plasmid pRW838 digested with NruI/EcoRI
and blunt-ended with Klenow to provide plasmid pC5AIPHA.
The plasmid pRW838 contains the rabies G gene (Kieny et al.,
1984) fused to the vaccinia H6 promoter in a canarypox

insertion plasmid (C5 locus). Digestion with <u>Nru</u>I and <u>Eco</u>RI
excises the rabies G gene leaving behind the 5'-most 100 bp
of the H6 promoter and the C5 flanking arms.

The plasmid pC5AIPHA was digested with <u>Sma</u>I and <u>Sac</u>I to
excise an 820 bp fragment containing the H6 promoter and the
5'-most 645 bp of the EIV (A1/Prague/56) coding sequence.
This fragment was co-inserted into pBS-SK digested with
<u>Hind</u>III and <u>Sma</u>I with a 1.1 kb <u>Sac</u>I/<u>Hind</u>III fragment from
pC3EIVAIPHA containing the remainder of the HA coding
sequence. The resultant plasmid was designated as
pBSAIPHAVQ.

Plasmid pBSAIPHAVQ was then linearized with <u>Spe</u>I and
<u>Sma</u>I. This 4.7 kb fragment was ligated to a 1.8 kb
<u>Spe</u>I/partial <u>Hinc</u>II fragment derived from pBSA2FHA. The
resultant pBS-based plasmid, containing the EIV
(A1/Prague/56) and (A2/Fontainebleau/79) HA genes in a head
to head configuration, was designated as pBSAIPA2FHAVQ.

A <u>Not</u>I/<u>Xho</u>I fragment (3.5 kb) derived from pBSAIP2FHAVQ
containing the two HA genes was isolated and inserted into
pSD542 (described below for EIV (A2/Suffolk/89) and pC5L to
provide the insertion plasmids pEIVHAVQVV and pEIVC5L,
respectively.

The C5L insertion plasmid was derived as follows.
Using the cosmid vector pVK102 (Knauf and Nester, 1982), a
genomic library for vCP65 (ALVAC-based rabies G recombinant
with rabies in C5 locus) was constructed. This library was
probed with the 0.9 kb <u>Pvu</u>II canarypoxvirus genomic fragment
contained within pRW764.5 (C5 locus). These canarypox DNA
sequences contain the original insertion locus. A clone
containing a 29 kb insert was grown up and designated
pHCOS1. From this cosmid containing C5 sequences, a 3.3 kb
<u>Cla</u> fragment was subcloned. Sequence analysis from this
<u>Cla</u>I fragment was used to extend the map of the C5 locus
from 1-1372.

The C5 insertion vector, pC5L, was constructed in two
steps. The 1535 bp left arm was generated by PCR
amplification using oligonucleotides C5A (SEQ ID NO:294)
(5'-ATCATCGAATTCTGAATGTTAAATGTTATACTTTG) and C5B (SEQ ID
NO:295) (GGGGGTACCTTTGAGAGTACCACTTCAG-3'). The template DNA

was vCP65 genomic DNA. This fragment was cloned into
EcoRI/SmaI digested pUC8. The sequence was confirmed by
standard sequencing protocols. The 404 bp right arm was
generated by PCR amplification using oligonucleotides C5C
(SEQ ID NO:296) (5'-ATCATCCTGCAGGTATTCTAAACTAGGAATAGATG-3')
and C5DA (SEQ ID NO:297) (5'-ATCATCCTGCAGGTATTC
TAAACTAGGAATAGATG-3'). This fragment was then cloned into
the vector previously generated containing the left arm
digested with SmaI/PstI. The entire construct was confirmed
by standard sequence analysis and designated pC5L. This
insertion plasmid enables the insertion of foreign genes
into the C5 locus.

**Example 45 - CONSTRUCTION OF INSERTION PLASMIDS TO
                GENERATE ALVAC- AND NYVAC-BASED RECOMBINANTS
                EXPRESSING INFLUENZA VIRUS (A2/SUFFOLK/89)
                HEMAGGLUTININ GENE**

     An M13 clone containing the hemagglutinin (HA) gene
from equine influenza virus (A2/Suffolk/89) was provided by
Dr. M. Binns (Animal Health Trust, P.O. Box 5, Newmarket,
Suffolk, CB8 7DW, United Kingdom). This clone contains a
full-length 1.7 kb cDNA fragment containing this HA gene
inserted into the M13 vector via the HindIII site.
     Initially, the equine influenza virus (EIV) HA gene was
amplified from the above M13 clone by PCR using
oligonucleotides EIVS1 (SEQ ID NO:298) (5'-ATGAAGACAACC
ATTATTTTG-3') and EIVS2 (SEQ ID NO:299) (5'-TCAAATGCAAA
TGTTGCATCT-3'). This 1.7 kb fragment was ligated into pBS-
SK (Stratagene, La Jolla, CA) digested with SmaI. Two
positive clones were derived and analyzed by nucleotide
sequence analysis (Goebel et al., 1990a). Clone A contained
one non-conserved base change while clone B contained three
such changes compared to the sequence provided in FIG. 25
(SEQ ID NO:300). To generate a full-length correct version
of the EIVHA gene, clone B was digested with SacI and MscI
to excise a 390 bp fragment. This fragment was ligated into
a 4.3 kb MscI/partial SacI fragment derived from clone A.
This provided a corrected EIVHA and was designated as
pBSEIVHS.
     The 5'-most 360 bp of the EIVHA coding sequence was
derived from pBSEIVHS by PCR using oligonucleotides I3L5EIV

(SEQ ID NO:301) (5'-GTTAATCATGAAGACAACCATTATTTTGATAC-3')
and EIVPVU (SEQ ID NO:302) (5'-AGCAATTGCTGAAAGCGC-3'). The
entire I3L promoter region (Goebel et al., 1990a,b) was
derived from pMPI3L101 by PCR using oligonucleotides I3L5B5
(SEQ ID NO:303) (5'-ATCATCGGATCCCGGGACATCATGCAGTGGTTAAAC-3')
and EIV5I3L (SEQ ID NO:304) (5'-CAAAATAATGGTTGTCTTCATGAT
TAAACCTAAATAATTGTAC-3'). These fragments were fused due to
the complementary conferred by the engineering of
oligonucleotides I3L5EIV (SEQ ID NO:301) and EIV5I3L (SEQ ID
NO:304) by PCR with oligonucleotides I3L5B5 (SEQ ID NO:303)
and EIVPVU (SEQ ID NO:302) to yield a 480 bp fragment.

Plasmid pMPI3L101 contains an expression cassette
consisting of the gene encoding the rabies glycoprotein
under the control of the I3L promoter, all inserted into a
vaccinia insertion plasmid deleted for ORFS C6L-K1L (Goebel
et al., 1990a,b). The I3L promoter consists of 101 bases
(nt 64,973-65,074 Goebel et al., 1990a,b) immediately
upstream from the initiation codon of the ORF I3L.

The above derived fusion fragment linking the I3L
promoter precisely to the 5' region of the EIVHA coding
sequence was digested with BamHI (5'-end) and AccI (3'-end)
and the 400 bp fragment isolated. This fragment was ligated
to a 4.6 kb BamHI/partial AccI fragment derived from
pBSEIVHS and the resultant plasmid designated as
pBSEIVHSI3L.

A 1.8 kb SmaI/XhoI fragment containing the EIVHA
expression cassette was derived from pBSEIVHSI3L. This
fragment was inserted into a SmaI/XhoI digested pSD542
(described in Example 32) insertion vector to yield
pTKEIVHSI3L.

The 1.8 kb SmaI/XhoI fragment from pBSEIVHEI3L (above)
was inserted into the CPpp (ALVAC) insertion plasmid,
VQCP3L, digested with SmaI/XhoI. The resultant plasmid was
designated as pC3EIVHSI3L.

Insertion plasmid VQCP3L was derived as follows.
VQCPCP3L was derived from pSPCP3L (defined in Example 32) by
digestion with XmaI, phosphatase treating the linearized
plasmid, and ligation to annealed, kinased oligonucleotides
CP23 (SEQ ID NO:305) (5'-CCGGTTAATTAATTAGTTATTAGACAAGG

TGAAAACGAAACTATTTGTAGCTTAATTAATTAGGTCACC-3') and CP24 (SEQ
ID NO:306) (5'-CCGGGGTCGACCTAATTAATTAAGCTACAAATAGTTTCGTTT
TCACCTTGTCTAATAACTAATTAATTAA-3').

**Example 46   -   DEVELOPMENT OF ALVAC-EQUINE INFLUENZA VIRUS
                   RECOMBINANTS**

Plasmid pEIVC5L contains the hemagglutinin coding
sequences for equine-1, A1/Prague/56 (H7) and equine-2,
A2/Fontainebleau/79 (H3). Both genes are linked to the
vaccinia virus H6 promoter and inserted at the de-orfed C5
locus. ALVAC virus was used as the rescuing virus in *in
vitro* recombination to rescue the inserted DNA. Positive
plaques were selected on the basis of hybridization to H7
and H3 coding sequences. Recombinant plaques were plaque
purified until a pure population containing both foreign
genes was achieved. At this time the recombinant was
declared vCP128 and a stock virus was established. .
Immunofluorescence analysis was performed using a monoclonal
antibody specific for the H3 hemagglutinin and a polyclonal
anti-H7 serum from horse. Surface fluorescence was detected
on vCP128 infected VERO cells using both reagents indicating
that both antigens were appropriately presented on the
infected cell surface.

Immunoprecipitation analysis using the H3 specific
monoclonal antibodies demonstrated the presence of a protein
of approximately 75 kd in vCP128 infected CEF cells. This
potentially represents the HA_0 precursor glycoprotein. No
cleavage products were detected. Immunoprecipitation
analysis using the H7 specific polyclonal serum demonstrated
the presence of a precursor glycoprotein of approximately 75
kd. The HA_1 and HA_2 cleavage products with molecular
weights of approximately 45 and 30 kd respectively were also
visualized.

Plasmid pC3EIVHS13L contains the hemagglutinin coding
sequence of the equine-2 A2/Suffolk/89 subtype. The gene is
linked to the vaccinia virus I3L promoter and inserted at
the de-orfed C3 insertion site. ALVAC virus was used as the
rescuing virus in *in vitro* recombination to rescue the
foreign gene. Recombinant plaques were selected on the
basis of hybridization to a H3 specific radiolabelled probe.

Positive plaques were plaque purified until a pure recombinant population was achieved. At this time the recombinant was declared vCP159 and a virus stock established. Immunofluorescence analysis on vCP159 infected CEF cells using an H3 specific serum polyclonal from chicken indicated that an immunologically recognized protein was expressed on the infected cell surface.

Example 47 - **DEVELOPMENT OF NYVAC RECOMBINANTS CONTAINING THE HEMAGGLUTININ GLYCOPROTEINS OF EQUINE INFLUENZA VIRUS SUBTYPES**

Plasmid pEIVHAVQVV contains the sequences encoding equine-1, A1/Prague/56 (H7) and equine-2 A2/Fontainebleau/79 (H3). Both genes are linked to the vaccinia virus H6 promoter and inserted at the TK site. NYVAC (vP866) was used as the rescuing virus in *in vitro* recombination to rescue the foreign genes. Recombinant plaques were selected on the basis of hybridization to radiolabelled H3 and H7 specific proteins. Recombinant progeny virus was plaque purified until a pure population was achieved. At this time the recombinant was declared vP961 and a virus stock established. Immunofluorescence analysis using a H3 specific monoclonal antibody and a polyclonal anti-H7 serum indicated that both glycoproteins were expressed on the infected cell surface. Immunoprecipitation analysis with the same reagents indicated that the H3 glycoprotein was expressed as a precursor glycoprotein with a molecular weight of approximately 75 kd. No cleavage products were evidenced. The H7 glycoprotein was evident as a precursor glycoprotein of approximately 75 kd and HA_1 and HA_2 cleavage products with molecular weights of approximately 45 and 30 kd, respectively.

Plasmid pTKEIVHSI3L contains the coding sequence of the equine-2 A2/Suffolk/89 hemagglutinin glycoprotein. The coding sequence is linked to the I3L promoter and inserted at the TK site. NYVAC (vP866) was used as the rescuing virus and recombinant plaques selected on the basis of hybridization to a H3 specific radiolabelled probe. Recombinant plaque progeny were plaque purified until a pure population was achieved. At this time the recombinant was declared vP1063 and a virus stock established.

Immunofluorescence analysis using a polyclonal anti-H3 serum
from chicken indicated that an immunologically recognized
protein was expressed on the infected cell surface.

Example 48 – **CONSTRUCTION OF VACCINIA VIRUS/FELV
                INSERTION PLASMIDS**

FeLV (Feline Leukemia Virus) _env_ DNA sequences were
supplied by Dr. F. Galibert (Laboratories d'Hematologie
Experimentale Hospital Saint-Luis, Paris, France) in the
form of a 2.4 kbp FeSV-SM DNA (Guilhot et al.., 1987)
fragment inserted into an M13mp8 vector (Messing, 1983).
This 2.4 bp _Pst_I/_Kpn_I fragment containing the entire open
reading frame (FeLV p70 + p13E) was isolated and inserted
into pUC18 (Messing, 1983) for convenience. The _Kpn_I site
at the 3' end of the _env_ sequences were converted to a _Pst_I
site and the 2.4 kbp _Pst_I fragment was isolated and ligated
into _Pst_I digested pTp15 (Guo et al., 1989). The resultant
plasmid was designated pFeLV1A.

In vitro mutagenesis (Mandecki, 1986) was used to
convert pFeLV1A to pFeLV1B. This was done using
oligonucleotide SPBGLD (SEQ ID NO:307) (5'-AATAAATCAC
TTTTTATACTAATTCTTTATTCTATACTTAAAAAGT-3'). Mutagenesis with
this oligonucleotide enabled the removal of the _Bgl_II site
at the border of the H6 promoter and HA sequences. This
provides the actual sequences of these DNA segments as found
in the virus.

Plasmid pFeLV1B was then mutagenized with
oligonucleotide FeLV5P (SEQ ID NO:308) (5'-CGCTATAGG
CAATTCAAACATAGCATGGAAGGTCCAAACGCACCCA-3') to create pFeLV1C.
In vitro mutagenesis was done as described by Mandecki
(1986) with the following modification. Following digestion
of pFeLV1B at the unique _Sma_I site, the DNA was digested
with _Bal_31. At times 5 sec., 10 sec., 20 sec., 40 sec., and
80 sec., aliquots were taken and the reaction terminated by
adding EGTA to a final concentration of 20mM. The aliquots
were pooled and used in the mutagenesis reaction. Resultant
plasmid, pFeLV1C, contained the FeLV _env_ gene juxtaposed 3'
to the vaccinia virus H6 promoter such that there exists and
ATG to ATG substitution. The plasmid, pFeLV1C, was used in
in vitro recombination tests with vP425 as the rescuing

virus to construct a recombinant vaccinia virus (vP453)
which expresses the entire FeLV envelope glycoprotein.

     The plasmid pFeLV1C was used as a reagent to generate
pFeLV1D. This recombinant plasmid contains the entire FeLV
env gene except it lacks the putative immunosuppressive
region (Cianciolo et al., 1985; Mathes et al., 1978). The
sequence encoding the immunosuppressive region (nucleotide
2252-2332 of sequence in Guihot et al., 1987) was deleted by
in vitro mutagenesis (Mandecki, 1986) in the following
manner. The plasmid, pFeLV1C, was linearized with BsmI.
The linearized plasmid was treated with Bal31 and aliquots
were taken at 1 min., 2 min., 4 min., and 8 min. and pooled
for use in the mutagenesis reaction. In vitro mutagenesis
was done using oligonucleotide FeLV1SD (SEQ ID NO:309)
(5'-ACCTCCCTCTCTGAGGTAGTCTATGCAGATCACACCGGACTCG
TCCGAGACAATATGGCTAAATTAAGAGAAAGACTAAAACAGCGGCAGCAACTGTTTGACT
CCCAACAG-3'). The resultant plasmid, pFeLV1D, was used in
in vitro recombination tests with vP410 as the rescuing
virus to generate vP456. This vaccinia virus recombinant
was generated to express the entire envelope glycoprotein
lacking the putative immunosuppressive region.

**Example 49 - CONSTRUCTION OF AVIPOXVIRUS/FeLV
                INSERTION PLASMIDS**

     For construction of the FP-1 recombinants, the 2.4 kbp
H6/FeLV env sequences were excised from pFELV1A (described
above) with BglII and by partial digestion with PstI. The
BglII site is at the 5' border of the H6 promote sequence.
The PstI site is located 420 bp downstream from the
translation termination signal for the envelope glycoprotein
open reading frame.

     The 2.4 kbp H6/FeLV env sequence was inserted into
pCE11 digested with BamH1 and PstI. The FP-1 insertion
vector pCE11, was derived from pRW731.13 by insertion of a
multiple cloning site into the nonessential HindII site.
This insertion vector allows for the generation of FP-1
genome. The recombinant FP-1/FeLV insertion plasmid was
then designated pFeLVF1. This FP-1/FeLV insertion plasmid
was then designated pFeLVF1. This construction does not
provide a precise ATG for ATG substitution.

To achieve the precise ATG:ATG construction, a NruI/SstII fragment of approximately 1.4 kbp was derived from the vaccinia virus insertion vector pFeLV1C (described herein). The NruI site occurs within the H6 promoter at a positive 24 bp upstream from the ATG. The SstII site is located 1.4 kbp downstream from the ATG and 1 kbp upstream from the translation termination codon. The NruI/SstII fragment was ligated to a 9.9 kbp fragment which was generated by digestion of pFeLVF1 with SstII and by partial digestion with NruI. This 9.9 kbp fragment contains the 5.5 kbp FP-1 flanking arms, the pUC vector sequences, 1.4 kbP of FeLV sequence corresponding to the downstream portions of the env gene, and the 5'-most portion (approximately 100 bp) of the H6 promoter. The resultant plasmid was designated pFeLVF2. The precise ATG:ATG construction was confirmed by nucleotide sequence analysis.

A further FP-1 insertion vector, pFeLVF3, was derived from FeLVF2 by removing the FeLV env sequences corresponding to the putative immunosuppressive region (described above). This was accomplished by isolating a PstI/SstII fragment of approximately 1 kbp obtained from the vaccinia virus insertion vector, pFeLV1D (described above), and inserting this fragment into a 10.4 kbp PstI/SstII fragment containing the remaining H6/FeLV env gene derived by digestion of pFeLVF2 with PstI and SstII.

The insertion plasmids, pFeLVF2 and pFeLVF3, were using in in vitro recombination tests with FP-1 as the rescuing virus. Progeny virus was plated on primary chick embryo fibroblast (CEF) monolayers obtained from 10 day old embryonated eggs (SPAFAS, Storrs, CT) and recombinant virus screened for by plaque hybridization on CEF monolayers. Recombinant progeny identified by hybridization analyses were selected and subjected to four round of plaque purification to achieve a homogeneous population. An FP-1 recombinant harboring the entire FeLV env gene has been designated vFP25 and an FP-1 recombinant containing designated vFP32.

For construction of the CP recombinants, a 2.2 bp fragment containing the H6/FeLV env sequences were excised

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from pFeLVF2 and pFeLVF3 by digestion with KpnI and HpaI.
The KpnI site is at the 5' border of the H6 promoter
sequence. The HpaI site is located 180 bp downstream from
the translation termination signal for the envelope
glycoprotein open reading frame. These isolated fragments
were blunt-ended. These 2.2 kbp H6/FeLV env sequences were
inserted into the nonessential EcoRI site of the insertion
plasmid pRW764.2 following blunt-ending of the EcoRI site.
This insertion vector enables the generation of CP
recombinants harboring foreign genes in the C3 locus of the
CP genome. The recombinant CP insertion plasmid was then
designated pFeLVCP2 and pFeLVCP3, respectively.

      The insertion plasmids, pFeLVCP2 and pFeLVCP3, were
used in in vitro recombination tests with CP as the rescuing
virus. Progeny of the recombination were plated on primary
CEF monolayers obtained from 10 day old embryonated eggs
(SPAFAS, Storrs, CT) and recombinant virus selected by
hybridization using radiolabeled FeLV DNA as a probe.
Positive hybridizing plaques were selected and subjected to
four rounds of plaque purification to achieve a homogeneous
population. A recombinant expressing the entire FeLV env
gene has been designated vCP35 and a recombinant expressing
the entire env gene lacking the immunosuppressive region was
designated vCP37.

**Example 50 - GENERATION OF AN ALVAC-BASED RECOMBINANT
                CONTAINING THE FeLV-B env GENE**

      Plasmid pFeLV env 24 was obtained from Rhone-Merieux
(Lyon, France) and contains the FeLV-B env gene. The
plasmid contains a 4.2 kb cDNA derived fragment derived from
the NCE161 FeLV strain. Plasmid pFeLV env 34 contains the
4.2 kb FeLV-B-specific insert in the SmaI site of pBS-SK
(Strategene, La Jolla, CA). The sequence of the FeLV-B env
gene is presented in FIG. 26 (SEQ ID NO:310). In this
sequence the initiation codon (ATG) and termination codon
(TGA) are underlined.

      The expression cassette for the FeLV-B env was
constructed as follows. The vaccinia virus H6 promoter was
derived from plasmid pI4LH6HIV3B (described herein with
respect to HIV) by PCR using oligonucleotides H65PH (SEQ ID

NO:164) (5'-ATCATCAAGCTTGATTCTTTATTCTATAC-3') and H63PFB
(SEQ ID NO:311) (5'-GGGTGCGTTGGACCTTCCATTACGATAGAAACTTA
ACGG-3'). Amplification of these sequences with these
oligonucleotides generated an H6 promoter with a 5' HindIII
site and a 3'-end containing the initial 20 bp of the FeLV-B
env coding sequence.

The 5'-portion of the FeLV-B env gene was derived by
PCR from pFeLV-B env 34 using oligonucleotides FB5P (SEQ ID
NO:312) (5'-CCGTTAAGTTTGTATCGTAATGGAAGGTCCAAGCG-3') and
FB5PA (SEQ ID NO:313) (5'-GGGTAAATTGCAAGATCAAGG-3'). This
PCR-derived fragment contains homology to the 3'-most 23 bp
of the H6 promoter (5'-end) and a unique ApaI site at
position 546. The H6 promoter was fused to the 5'-end of
the FeLV-B env gene by PCR using the two PCR fragments
defined above as template and oligonucleotides FB5PA (SEQ ID
NO:313) and H65PH (SEQ ID NO:164). The PCR fusion fragment
was digested with HindIII and ApaI to yield a 680 bp
fragment.

Plasmid pFeLV-B env 34 was digested with ApaI and NcoI
to liberate a 740 bp fragment containing the middle portion
of the env gene (FIG. 26). The 3'-end of the gene was
derived by PCR using pFeLV-b env 34 as template and
oligonucleotides FBT5D (SEQ ID NO:314) (5'-CCCCATGCCATTT
CCATGGCAGTGCTCAATTGGACCTCTGATTTCTGTGTCTTAATAG-3') and FB3PX
(SEQ ID NO:315) (5'-ATCATCTCTAGAATAAAAAATCATGGTCGGTCCG
GATC-3'). PCR amplification of the 3' portion of the env
gene with these oligonucleotides eliminated a T5NT element
at position 1326-1332. This sequence was altered by making
a T to C substitution at position 1329 (FIG. 26). This
change does not alter the amino acid sequence of the env
gene product. The 3'-end of the gene was engineered with an
XbaI site and a T5NT sequence. The 5'-end of this PCR-
derived fragment also contains a unique NcoI site
(corresponds with that at position 1298; FIG. 26). This
fragment was digested with NcoI and XbaI to generate a 707
bp fragment.

The 740 bp NcoI/ApaI and 707 bp NcoI/XbaI fragments
were co-inserted into pBS-SK digested with ApaI and XbaI.
The resultant plasmid was designated as pBSFB3P. The 1.5 kb

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ApaI/XbaI fragment from pBSFB3P was then co-inserted into
pBS-SK, digested with HindIII and XbaI, with the 680 bp PCR
fragment containing the H6 promoted 5'-end of the FeLV-B env
gene (above). The resultant plasmid was designated as
pB5FEB.

The 2.2 kb HindIII/XbaI fragment from pB5FEB,
containing the H6 promoted FeLV-B env gene, was isolated and
blunt-ended with the Klenow fragment. This blunt-ended
fragment was inserted into pC5L (see discussion regarding
HIV herein) digested with SmaI to yield pC5LFEB.

Plasmid pC5LFEB was used in standard in vitro
recombination assays with ALVAC(CPpp) as the rescue virus.
Recombinant plaques were identified using FeLV-B env
specific DNA probes. Following three round of plaque
purification, the virus was propagated and designated as
vCP177.

**Example 51 - GENERATION OF AN ALVAC-FeLV-A ENV
                 RECOMBINANT VIRUS**

The plasmid pFGA-5 from which the FeLV-A env sequences
were derived was provided by Dr. J. Neil (University of
Glasgow) and described previously (Stewart et al., 1986).
Initially, the 531 bp PstI/HindIII fragment corresponding to
nucleotides 1 to 531 (Stewart et al., 1986) was excised and
ligated into pCPCV1 digested with PstI and HindIII and
designated as pC3FA-1. The plasmid pCPCV1 was derived as
follows. Plasmid pFeLV1A was digested with PstI to excise
the FeLV sequences and religated to yield plasmid pFeLVF4.
The vaccinia virus H6 promoter element (Taylor et al., 1988)
followed by a polylinker region were liberated from pFeLVF4
by digestion with KpnI and HpaI. The 150 bp fragment was
blunt-ended using T4 DNA polymerase and inserted into
pRW764.2, a plasmid containing a 3.3kb PvuII genomic
fragment of canarypox DNA. pRW764.2 was linearized with
EcoRI, which recognizes a unique EcoRI site within the
canarypox sequences, and blunt-ended using the Klenow
fragment of the E. coli DNA polymerase. The resultant
plasmid was designated as pCPCV1. This plasmid contains the
vaccinia virus H6 promoter followed by a polylinker region
and flanked by canarypoxvirus homologous sequences.

The plasmid pC3FA-1 was linearized with PstI and mutagenized in an *in vitro* reaction via the Mandecki procedure (1986) using oligonucleotide FENVAH6-1 (SEQ ID NO:316) (5'-CCGTTAAGTTTGTATCGTAATGGAAAGTCCAACGCAC-3'). The *in vitro* mutagenesis procedure removed extraneous 5'-noncoding sequences resulting in a precise ATG:ATG configuration of the vaccinia H6 promoter element and the FeLV-A env sequences. The resultant plasmid was designated as pH6FA-1.

The remainder of the FeLV-A env gene was derived from pFGA-5 by standard PCR using custom synthesized oligonucleotides (Applied Biosystems, San Rafael, CA). An 836 bp PCR fragment was derived using pFGA-5 as template and the oligonucleotides FENVA-2 (SEQ ID NO:317) (5'-CCATAATTCG ATTAAGACACAGAATTCAGAGGTCCAATTGAGCACC-3') and FENVAH (SEQ ID NO:318) (5'-CAAGATGGGTTTTGTGCG-3'). This fragment corresponds to nucleotides 488 to 1327 of the FeLV-A env gene (Stewart et al., 1986). The use of oligonucleotide FENVA-2 (SEQ ID NO:317) alters the nucleotide sequence at positions 1301 to 1309 from GAT_5GT to GAATTCTGT. This alteration eliminates the T5NT sequence motif known to be recognized as a poxvirus early transcription termination signal (Yuen and Moss, 1987) and introduces an EcoRI restriction site at this position. These nucleotide manipulations change amino acid 414 from glutamic acid to the conserved amino acid aspartic acid (Stewart et al., 1986). Amino acid 415 is not altered by these nucleotide changes. This 836 bp fragment was digested with HindIII and EcoRI to generate a 770 bp fragment corresponding to nucleotides 532 to 1302 of the FeLV-A env gene.

A 709 bp PCR fragment was derived using pFGA-5 as template and the oligonucleotides FENVA-3 (SEQ ID NO:319) (5'-GGTGCTCAATTGGACCTCTGAATTCTGTGTCTTAATCGAATTATGG-3') and FENVA-4 (SEQ ID NO:320) (5'-ATCATCAAGCTTTCATGGTCGGTCCGG-3'). This fragment corresponds to nucleotides 1281 to 1990 of the FeLV-A env gene (Stewart et al., 1986). Using oligonucleotide FENVA-3 (SEQ ID NO:319) to amplify this fragment also alters the T5NT element and introduces an EcoRI site as above for the 836 bp PCR derived fragment.

The 709 bp fragment was digested with EcoRI/HindIII and the
resultant fragment was co-inserted with the 770 bp
HindIII/EcoRI fragment, derived from the 836 bp fragment
(above), into pBS-SK (Stratagene, La Jolla, CA) digested
with HindIII. The resultant plasmid was designated as
pF3BS1-B and the FeLV env sequences were confirmed by
nucleotide sequence analysis.

     To reconstruct the entire FeLV-A env gene linked
precisely to the vaccinia virus H6 promoter, a 1.5 kb
HindIII fragment was isolated from pF3BS1-B. This fragment
corresponds to nucleotides 532 to 1990 of the FeLV-A env
(Stewart et al., 1986). The 1.5 kb HindIII fragment was
ligated to pH6FA-1 digested with HindIII. Plasmid
constructs containing the 1.5 kb HindIII fragment were
screened for the proper orientation by restriction analysis
and a plasmid clone containing the entire intact FeLV-A env
gene linked to the H6 promoter was designated as pH6FA-3.

     The 2.2 kb H6/FeLV-A env expression cassette was
excised from pH6FA-3 by partial digestion with EcoRI
followed by a partial digestion with HindIII. The fragment
was inserted into pRW831 digested with HindIII and EcoRI.
The resultant plasmid was designated as pC5FA.

     pRW831 refers to an ALVAC (CPpp) insertion plasmid
which enables the insertion of foreign genes into the C5
open reading frame. In the process of insertion into this
region, the use of pRW831 causes the deletion of most of the
C5 open reading frame. To generate pRW831 the following
manipulations were done. An 880 bp PvuII genomic fragment
from the canarypoxvirus genome was inserted between the
PvuII sites of pUC9. The canarypox sequences contained
within the resultant plasmid, pRW764.5, was analyzed by
nucleotide sequence analysis and the C5 open reading frame
was defined. Previously, insertion between a pair of BglII
sites situated within the C5 ORF was used to engineer
recombinants at this locus (Taylor et al., 1992). The
sequence of the entire region is provided in FIG. 16 (SEQ ID
NO:220). The nucleotide sequence begins (SEQ ID NO:220) at
the PvuII site. The C5 ORF initiates at position 166 and
terminates at nucleotide 487. Precise manipulation of these

sequences enabled the deletion of nucleotides 167 through
455. Such a deletion was made so as not to interrupt the
expression of other viral genes.

The procedure to derive pRW831 is as follows. pRW764.5
was partially digested with RsaI and the linearized fragment
was isolated. The RsaI linear fragment was redigested with
BglIII. The resultant 2.9 kb RsaI/BglII fragment (deleted
of nucleotides 156 through 462) was isolated and ligated to
annealed oligonucleotides RW145 (SEQ ID NO:107) and RW146
(SEQ ID NO:108). The resultant plasmid was designated as
pRW831 and contains a sequence with unique HindIII, SmaI,
and EcoRI sites in place of the C5 sequences.

Plasmid pC5FA was used in recombination experiments
with ALVAC(CPpp) as the rescuing virus. Recombinant viruses
were identified by in situ plaque hybridization using a
radiolabeled FeLV-A env-specific probe. Following three
cycles of plaque purification with subsequent hybridization
confirmation, the recombinant was designated as vCP83.

**Example 52   -   GENERATION OF AN ALVAC-FeLV-A ENV RECOMBINANT
                    VIRUS LACKING THE PUTATIVE IMMUNOSUPPRESSIVE
                    REGION OF p15E**

The putative immunosuppressive region is situated
within the p15E transmembrane region of the FeLV envelope
glycoprotein (Cianciolo et al., 1986; Mathes et al., 1978).
This region was deleted in the following manner. The FeLV-A
env sequences from nucleotide 1282 to 1602 (Stewart et al.,
1986) were amplified by PCR from pFGA-5 using
oligonucleotides FENVA-3 (SEQ ID NO:320) and IS-A (SEQ ID
NO:468) (5'-TAAGACTACTTCAGAAAG-3'). The env sequences from
nucleotide 1684 to 1990 (Stewart et al., 1986) were
amplified by PCR from pFGA-5 using oligonucleotides FENVA-4
(SEQ ID NO:320) and IS-B (SEQ ID NO:323) (5'-GCGGATCACA
CCGGACTC-3'). The former PCR-derived fragment was digested
with EcoRI and the latter with HindIII and was subsequently
kinased with ATP and T4 kinase. These fragments were co-
ligated into pBS-SK digested with HindIII and EcoRI. The
resultant plasmid was confirmed by nucleotide sequence
analysis and designated as pBSFAIS⁻. Ligation of the above
fragments joins the sequences 5' and 3' to the 81 bp DNA
segment encoding the putative immunosuppressive region and,

therefore, deletes the sequences encoding the
immunosuppressive peptide.

The FeLV-A sequences lacking the region encoding the
immunosuppressive region were excised from pC5FA by
digestion with SstII/ApaI. This 381 bp fragment was
replaced by the 300 bp SstII/ApaI fragment from pBSFAIS⁻.
The ligation that was done was with a 4.8kb SstII/ApaI
fragment from pC5FA and the 300 bp fragment described above.
The resultant plasmid was designated pC5FAISD.

The plasmid pC5FAISD was employed in recombination
experiments with ALVAC (CPpp) as the rescuing virus.
Recombinant viruses were identified by *in situ* hybridization
using a radiolabeled FeLV-A env specific probe. Following
three cycles of plaque purification, the recombinant was
designated as vCP87. This recombinant contains the FeLV-A
env gene lacking the region encoding the putative 27 amino
acid immunosuppressive region. The gene was inserted into
the C5 locus.

**Example 53 - GENERATION OF ALVAC-FeLV-A gag
                RECOMBINANT VIRUSES**

The FeLV-A gag/pol sequences were derived from plasmid
pFGA-2 gag. This plasmid was derived from the FeLV-A
infectious clone pFGA-2 (Stewart et al., 1986) by subcloning
the 3.5 kb PstI subfragment containing a portion of the LTR
(651 bp) sequences, the entire gag gene, and 1272 bp of the
pol gene. The 3.5 kb fragment was inserted into PstI
digested pUC8 (Bethesda Research Laboratories, Gaithersburg,
MD). Initially, this 3.5 kb PstI FeLV-A DNA fragment was
isolated and inserted into pBS-SK (Stratagene, La Jolla,
CA). The resultant plasmid was designated as pBSGAG. The
entire 3.5 kb insert was analyzed by nucleotide sequence
(SEQ ID NO:324) (FIG. 27) analysis to confirm position of
the initiation codon (nucleotide 652 to 654 underlined in
FIG. 27) and pertinent restriction sites defined in the
nucleotide sequence of the gag region previously reported
for FeLV-B (Leprevotte et al., 1984).

The plasmid pFGA-2 gag was digested with BglII and PstI
to liberate a 2.5 kb fragment. BglII recognizes a site at
nucleotide position 1076 (SEQ ID NO:324) while PstI

recognizes a site at the end of the FeLV-A insert. The 2.5 kb fragment was isolated and redigested with HindIII and PstI which recognize sites within the co-migrating plasmid sequences. This eliminated the ability of the plasmid sequences to compete in subsequent ligation reactions.

PCR was used to derive the 5' portion of the FeLV-A gag coding sequences. The plasmid pFGA2 gag was used as template with oligonucleotides FGAGBGL (SEQ ID NO:325) (5'-GATCTCCATGTAGTAATG-3') and FGAGATG (SEQ ID NO:326) (5'-CGATATCCGTTAAGTTTGTATCGTAATGTCTGGAGCCTCTAGTG). Oligonucleotide FGAGATG (SEQ ID NO:326) contains the 3'-most 25 nucleotides of the vaccinia virus H6 promoter and includes the 3'-most 3 bp of the NruI site at its' 5'-end. These H6 sequences are precisely joined at the ATG (initiation codon) and the nucleotides corresponding to the initial 16 nucleotides of the gag coding sequence. . Oligonucleotide FGAGBGL (SEQ ID NO:325) corresponds to the reverse complement of sequences 59 bp downstream from the unique BglII site in the gag sequences (Leprevotte et al., 1984). PCR using these reagents yielded a 500 bp fragment which was subsequently digested with BglII to generate a 450 bp fragment.

The 450 bp BglII digested PCR-derived fragment was coligated with the 2.5 kb BglII/PstI fragment, containing the remainder of the gag gene and a portion of the pol gene, and pCPCV1 (above in env construction) digested with NruI and PstI. pCPCV1 (NruI/PstI) contains the 5' portion of the vaccinia virus H6 promoter including the 5'-most 3 bp of the NruI recognition signal. The resultant plasmid was designated as pC3FGAG.

The plasmid pC3FGAG was linearized with PstI, blunt-ended with T4 DNA polymerase and ligated to a 100 bp SspI/SmaI fragment excised from pSD513 (defined in Example 7). The 100 bp SspI/SmaI fragment provides termination codons at the 3' end of the FeLV-A gag/pol sequences. The resultant plasmid was designated as pC3FGAGVQ.

The FeLV-A gag/pol expression cassette was excised from pC3FGAGVQ by digestion with EcoRI and HindIII. The resultant 3.4 kb fragment was isolated and ligated with pC3I

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(defined in Example 32) digested with EcoRI and HindIII to
yield pC3D0FGAGVQ.

     The plasmid pC3D0FGAGVQ was used in in vitro
recombination experiments with vCP83 and vCP87 as rescue
viruses. The recombinant containing the FeLV-A gag/pol
sequences and the entire FeLV-A env gene was designated as
vCP97 while the recombinant containing the same gag/pol
sequences and the entire FeLV-A env lacking the
immunosuppressive region was designated vCP93.

**Example 54 - INSERTION OF FeLV-A gag INTO A VACCINIA
                VIRUS BACKGROUND**

     The insertion plasmid pCEN151 was generated by cloning
a 3.3 kb EcoRI/HindIII fragment from pC3FGAG (above) into
the SmaI site of pSD553. This insertion was performed
following blunt-ending the fragment with the Klenow fragment
of the E. coli DNA polymerase in the presence of 2mM dNTPs.

     Plasmid pSD553 is a vaccinia deletion/insertion plasmid
of the COPAK series. It contains the vaccinia K1L host
range gene (Gillard et al., 1986; Perkus et al., 1990)
within flanking Copenhagen vaccinia arms, replacing the ATI
region (ORFS A25L, A26L; Goebel et al., 1990a,b). pSD553
was constructed as follows. The polylinker region located
at the vaccinia ATI deletion locus of plasmid pSD541
(defined in Example 10) was expanded as follows. pSD541 was
cut with BglII/XhoI and ligated with annealed complementary
synthetic deoxyoligonucleotides MPSYN333 (SEQ ID NO:329)
(5'-GATCTTTTGTTAACAAAAACTAATCAGCTATCGCGAATCGATT
CCCGGGGGATCCGGTACCC-3') and MPSYN334 (SEQ ID NO:330)
(5'-TCGAGGGTACCGGATCCCCCGGGAATCGATTCGCGATAGCTGATTAG
TTTTTGTTAACAAAA-3') generating plasmid pSD552. The K1L host
range gene was isolated as a 1 kb BglII(partial)/HpaI
fragment from plasmid pSD452 (Perkus et al., 1990). pSD552
was cut with BglII/HpaI and ligated with the K1L containing
fragment, generating pSD553.

     Plasmid pCEN151 was used in in vitro recombination
experiments with vP866 as rescue virus to generate vP1011.

**Example 55 - IMMUNOFLUORESCENCE AND IMMUNOPRECIPITATION
                ANALYSIS OF FeLV ENV AND gag GENES IN ALVAC
                RECOMBINANT VIRUSES**

     Immunoprecipitation. Vero cell monolayers were

infected at an m.o.i. equal to 10 pfu/cell with parental or
recombinant viruses. At 1 hr post-infection, the inoculum
was aspirated and methionine-free medium supplemented with
(^{35}S)-methionine (DuPont, Boston, MA; 1000 Ci/mmol), 20
μCi/ml was added and further incubated till 18 hr post-
infection. Immunoprecipitation and immunofluorescence
analyses were performed as described previously (Taylor et
al., 1990) using a bovine anti-FeLV serum (Antibodies, Inc.,
La Jolla, CA) or a monoclonal antibody specific for the p27
core protein (provided by Rhone-Merieux, Inc., Athens, GA).

     FeLV Virus Isolation. On day one, 3x10^4 QN10
cells/well were plated in a 12-well plate in 1 ml of
Dulbecco's MEM containing HEPES buffer (DFB), 10% FBS, and 4
μg/ml polybrene. The cells were incubated overnight at
37°C. Without removing the medium, 200 μl of sample (cat
plasma) was added to each well. Following a 2 hr incubation
at 37°C, the medium as replaced with 1.5 ml of fresh DFB and
allowed to further incubate at 37°C. On day five, plates
were examined for transformation. If negative, medium was
replaced with 1.5 ml of fresh DFB and again allowed to
further incubate at 37°C. On day eight, plates were re-
examined for transformation. If negative, cells were
subcultured in 5 cm plates by dispersing cells by two washes
with trypsin-EDTA and placing in 4 ml DFB for inoculation
into a 5 cm plate. Cells were allowed to incubate for four
days at 37°C prior to examination for transformation.

     Detection of FeLV Antigen By Immunofluorescence. Blood
smears were fixed for five min in MeOH at -20°C, washed in
dH$_2$O, and then air dried. A volume of 24 μl of rabbit anti-
FeLV antibody was applied to the blood smear within a circle
inscribed on the smear with a diamond pen. The smear was
incubated in the presence of the antibody for 1 hr at 37°C
in a humidified chamber prior to washing three times with
PBS and one time with dH$_2$O. The smear was then air dried.
A volume of 25 μl of goat anti-cat IgG-FITC was applied to
the circle and incubated as above with the primary
antiserum. The sample was washed and dried as above for the
primary antiserum prior to examination for
immunofluorescence in a microscope with a ultra violet light

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source.

FeLV Antibody Neutralization Assay. On day one, 5×10^4
QN10 cells/well were plated in a 12-well plate in 1 ml DFB
plus 4 μg/ml polybrene. The cells were inoculated at 37°C.
Serum dilutions were prepared in round bottom 96 well plates
from 1:2 to 1:256 using 50 μl volumes of Leibowitz medium.
Added 50 μl FeLV-A at 4×10^5 focus forming units (ffu) per
ml. Two wells were included with medium without serum as a
virus control. Plates were incubated for 2 hr at 37°C.
Following the 2 hrs adsorption period, 25 μl of each
dilution was placed into a well of QN10 cells. Virus
control was titrated by diluting 1:2, 1:4, 1:8, and 1:6 in
50 μl volumes of Leibowitz medium in the 96-well plate prior
to inoculation of QN10 cells with 25 μl onto QN10 cells.
Plates were inoculated at 37°C for three days. On day day,
medium was replaced with 1 ml of DFB/well. Two days later,
foci were counted under a microscope. Neutralizing antibody
titers were estimated as the dilution of serum producing 75%
reduction in focus count compared to virus control.

In order to determine whether the env gene product
expressed by vCP83 and vCP87 was transported to the plasma
membrane of infected cells, immunofluorescence experiments
were performed as described previously (Taylor et al.,
1990). Primary CEF monolayers were infected with parental
(ALVAC) or recombinant viruses, vCP83 and vCP87 and
immunofluorescence was performed at 24 hr post-infection
using a bovine anti-FeLV serum. The results demonstrate
that cells infected with vCP83 showed strong surface
fluorescent staining, whereas cells infected with vCP87 or
parental ALVAC virus showed no significant surface staining.

Expression of the FeLV env gene product was also
analyzed in immunoprecipitation assays using the bovine
anti-FeLV serum. No FeLV-specific protein species were
precipitated from lysates derived from uninfected CEFs or
CEFs infected with the parental ALVAC virus. Three FeLV-
specific proteins were precipitated from vCP83 infected
cells with apparent molecular weights of 85 kDa, 70 kDa, and
15 kDa. This result is consistent with expression of the
precursor env gene product (85 kDa) and the mature cleavage

products p70 and p15E. Immunoprecipitation from lysates
derived form vCP88 infected cells demonstrated a single
FeLV-specific protein species with an apparent molecular
weight of 83 kDa. This is consistent with expression of a
non-proteolytically processed _env_ gene product of the size
expected following deletion of the putative
immunosuppressive region. So, in short, expression of the
env lacking the immunosuppressive region was apparently not
properly transported to the surface of infected cells nor
was it proteolytically cleaved to mature _env_ specific
protein forms.

      Expression of the FeLV _gag_-specific gene products was
analyzed by immunoprecipitation using monoclonal antibodies
specific to an epitope within the p27 core protein (D5) and
the bovine anti-FeLV serum. No FeLV-specific proteins were
precipitated from lysates derived from uninfected cells or
cells infected with parental viruses. Clearly, from vP1011,
vCP93, and vCP97 infected cells, FeLV specific protein
species of 55kDa were precipitated with the D5 and bovine
anti-FeLV serum. Protein species of these apparent
molecular weights are consistent with _gag_-specific precursor
forms. Low levels of a 27 kDa protein species consistent
with the size of the mature p27 core protein were also
apparent.

Example 56 - *IN VIVO EVALUATION OF vCP93 AND vCP97*

      The protective efficacy of vCP93 and vCP97 were
evaluated by a live FeLV challenge of cats following two
inoculations of the recombinant viruses. The ALVAC-based
FeLV recombinants were administered via the subcutaneous
route with 10^8 PFU on days 0 and 28. Cats were challenged
by an oronasal administration of an homologous FeLV-A strain
(Glasgow-1) at seven days following the booster inoculation.
Blood samples were obtained pre- and post-challenge for
evaluation of FeLV antigenemia (p27 detection), FeLV
isolation, the presence FeLV antigen in white blood cell
(WBC) smears by immunofluorescence, and the induction of
FeLV neutralizing activity.

      No adverse reactions were observed following
vaccination with the ALVAC (canarypoxvirus)-based

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recombinant viruses, vCP93 and vCP97. All six non-
vaccinated controls succumbed to the FeLV challenge and
developed a persistent viremia by three weeks following the
challenge exposure. This was evidenced by detection of p27
antigen in the blood, FeLV isolation and detection of FeLV
antigen by immunofluorescent analysis of WBC smears (Table
32). The non-vaccinated controls remained persistently
infected for the remainder of the study (until 12 weeks
post-challenge).

A persistent viremia developed in three of six cats
vaccinated with vCP93 at three weeks post-challenge. At
this timepoint, blood samplings from these three cats were
shown to contain p27 antigen and/or live FeLV (Table 32).
One of these cats (No. 2) resolved this infection by six
weeks post-infection and remained free of viremia through 12
weeks post-challenge. The other two cats (No. 1 and 5)
remained persistently infected by the three criteria, p27
antigenemia, FeLV isolation, and FeLV antigen detection in
WBCs. Three of six cats vaccinated with vCP93 (No. 3, 4,
and 6) were free of persistent viremia through nine weeks
post-challenge (Table 32). Two of these cats (No. 3 and 6)
remained free of circulating virus through week 12 post-
challenge, while one cat (No. 4) became suddenly infected at
12 weeks. So, partial protection (three of six cats) was
afforded protection against persistent viremia by
vaccination with vCP93.

Most impressively, all six cats vaccinated with vCP97
were fully protected against the homologous challenge with
FeLV-A (Glasgow-1). Only one of these cats (No. 12)
evidenced any suggestion of persistent viremia. This
occurred at three weeks post-challenge when p27 antigen was
detected in the blood sample (Table 32). No live FeLV was
ever isolated from the blood of this cat following
challenge. All other cats were free of p27 antigenemia,
free of live FeLV, and never demonstrated any FeLV antigen
in WBC smears for 12 weeks following challenge exposure
(Table 32).

Evolution of FeLV Neutralizing Antibodies. Due to the
potential role of neutralizing antibodies in protection

against FeLV infection (Russell and Jarrett, 1978; Lutz et al., 1980), the generation of such a response was monitored pre- and post-challenge. None of the cats in the study, whether vaccinated with vCP93 or vCP97, demonstrated any neutralizing antibody titers prior to FeLV challenge (Table 33). Following challenge, none of the cats which developed a persistent viremia had any detectable neutralizing antibody titers. Significantly, cats protected against a persistent infection developed FeLV-specific serum neutralizing titers (Table 33). These titers increased in magnitude in all protected cats following challenge, with the highest level being observed at the terminal time point of the study, at 12 weeks post-challenge (Table 33).

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Table 32.

Response of cats to challenge with feline leukemia virus

	Group	Cat No.	-5 E[1]V[2]	-2 EV	0 EV	+3 EV	+6 F[3]EV		+9 FEV	+12 FEV
1.	vCP 93:	1	--	--	--	++	⁻++	+++	+++	
	Felv-A	2	--	--	--	⁻+	---		---	---
	env(IS-)	3	--	--	--	--	---		---	---
	+gag/pol	4	--	--	--	--	---		---	++⁻
		5	--	--	--	++	⁻++	+++	+++	
		6	--	--	--	--	---		---	---
2.	vCP 97:	7	--	--	--	--	---		---	---
	Felv-A .	8	--	--	--	--	---		---	---
	env(IS+)	9	--	--	--	--	---		---	---
	+gag/pol	10	--	--	--	--	---		---	---
		11	--	--	--	--	---		---	---
		12	--	--	--	+⁻	---		---	---

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Table 32.   (continued)

| Group | Cat No. | Time (weeks) relative to challenge | | | | | | | |
		-5 E^1V^2	-2 EV	0 EV	+3 EV	+6 F^3 EV		+9 FEV	+12 FEV
3. Unvaccinated Controls	13	--	--	--	+ +	⁻+ +	+ + +	+ + +	
	14	--	--	--	+ +	⁻+ +	+ + +	+ + +	
	15	--	--	--	+ +	+ + +	+ + +	+ + +	
	16	--	--	--	+ +	⁻+ +	+ + +	+ + +	
	17	--	--	--	+ +	⁻+ +	+ + +	+ + +	
	18	--	--	--	+ +	⁻+ +	+ + +	+ + +	

*E = FeLV p27 antigen in plasma (ELISA)

V = infectious virus in plasma (virus isolation)

F = FeLV antigen in blood smears (immunofluorescence)

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Table 33.

FeLV neutralizing antibody titers[1]

Group	Cat. No.	Weeks Relative to Challenge						
		-5	-2	0	+3	+6	+9	+12
vCP93	1	0	0	0	0	0	0	0
	2	0	0	0	0	0	4	>32
	3	0	0	0	0	0	4	16
	4	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
	6	0	0	0	0	0	4	>32
vCP97	7	0	0	0	0	0	4	16
	8	0	0	0	0	0	0	>32
	9	0	0	0	0	0	4	8
	10	0	0	0	0	0	4	16
	11	0	0	0	0	0	4	8
	12	0	0	0	0	0	4	>32
Unvaccinated Controls	13	0	0	0	0	0	0	0
	14	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0
	16	0	0	0	0	0	0	0
	17	0	0	0	0	0	0	0
	18	0	0	0	0	0	0	0

[1] expressed as dilution of serum producing 75% reduction in focus count.

**Example 57 - GENERATION OF ALVAC-BASED RECOMBINANT
                EXPRESSING THE FHV-1 gD GLYCOPROTEIN**

Expression of the feline herpesvirus type 1 (FHV-1) gD
glycoprotein was accomplished by insertion of the FHV-1 gD
homolog gene under the control of the vaccinia virus I3L
promoter into the ALVAC vector. The donor plasmid required
to insert the cassette I3L-FHV-1 gD into ALVAC was generated
as follows.

The FHV-1 C0 strain genomic DNA was completely digested
with EcoRI and the fragment M (4470 bp) was excised from the
agarose gel (Geneclean procedure) and cloned into vector
pBS-SK⁺ digested with EcoRI and phosphatased. The resulting
plasmid containing the FHV-1 EcoRI M fragment was designated
pHFeM2. The FHV-1 EcoRI M fragment complete nucleotide
sequence for both strands was obtained from several
subclones of the FHV-1 EcoRI M fragment inserted into vector
pBS-SK⁺, using the modified T7 enzyme Sequenase (U.S.
Biochemical Corp.) (Tabor and Richardson, 1987). Standard
dideoxynucleotide chain termination reactions (Sanger et
al., 1977) were performed using double-stranded templates
that had been denatured in 0.4 M-NaOH (Hattori and Sakaki,
1986). The M13 forward and reverse primers were used to
obtain the initial sequence of each clone. Custom primers
(18 mer), synthesized by using standard chemistries
(Biosearch 8700 and Applied Biosystems 380B) were used for
subsequent sequence reactions. Sequences of the junctions
between consecutive fragments were confirmed on the initial
clone, pHFeM2. The PC\GENE (Intelligenetics) and IBI Pustell
software packages were used in all sequence data analyses.
Homology searches were done with the FASTP program (Lipman
and Pearson, 1985) against Swissprot release 18.0
(Intelligenetics). The full sequence of the FHV-1 C0 strain
gD homolog gene is shown in FIG. 28 (SEQ ID NO:290).

The 185 bp 5'-most region of the FHV-1 gD coding
sequence was derived using the plasmid pHFeM2 as template
and oligonucleotides JCA234 (SEQ ID NO:331) (5'-ATGATGACA
CGTCTACATTTT-3') and JCA235 (SEQ ID NO:332) (5'-TGTTACA
TAACGTACTTCAGC-3') and digested with BamHI. This 185 bp
fragment was fused to a 120 bp PCR-derived fragment

containing the I3L promoter element obtained using plasmid
pMP691 (I3L101RAB) and oligonucleotides MP287 (SEQ ID
NO:226) (5'-GATTAAACCTAAATAATTGT-3') and JCA158 (SEQ ID
NO:225) (5'-TTTTTCTAGACTGCAGCCCGGGACATCATGCAGTGGTTAAAC-3')
and digested with XbaI. The 185 bp and 120 bp fragments
were ligated together into vector pBS-SK$^+$ digested with XbaI
and BamHI to produce plasmid pJCA071. Sequences of the I3L
promoter, of the junction I3L-ATG and of the FHV-1 gD 5'-
most region were confirmed by direct sequencing of pJCA071.
Plasmid pJCA067 is a subclone of FHV-1 EcoRI M fragment. It
has been generated as follows. Plasmid pHFeM2 was digested
with BamHI and the 1850 bp BamHI-BamHI fragment was excised
from the agarose gel and ligated into pBS-SK$^+$ digested with
BamHI. This clone contains the 3'-region of FHV-1 gD and
sequences further downstream. Plasmid pJCA067 was digested
with BamHI and XhoI to excise the 1270 bp BamHI-FHV-1 gD 3'-
region-gI 5'-region-XhoI fragment. This fragment was
ligated into vector pBS-SK$^+$ digested with BamHI and XhoI to
produce pJCA072. The 3'-most 290 bp of the FHV-1 gD coding
sequence was derived by PCR using plasmid pHFeM2 as template
and oligonucleotides JCA237 (SEQ ID NO:333) (5'-AATTTT
CTCGAGAAGCTTGTTAACAAAAATCATTAAGGATGGTAGATTGCATG-3') and
JCA242 (SEQ ID NO:334) (5'-GAGGATTCGAAACGGTCC-3').
Synthesis of this fragment with these custom synthesized
oligonucleotides 1) contains the 3' most sequences of FHV-1
gD and XbaI site at the 5'end, and 2) adds a T$_5$NT element
and unique HpaI, HindIII and XhoI sites at the 3'end of the
FHV-1 gD coding sequence. This fragment was digested with
XbaI and XhoI and ligated with the 3575 bp XbaI-XhoI
fragment obtained from plasmid pJCA072 digested with XbaI
and XhoI. The resulting plasmid was designated pJCA073.

     Sequence of the 290 bp XbaI-->XhoI portion of FHV-1 gD
was confirmed by direct sequencing of pJCA073. Plasmid
pJCA071 was digested with SmaI and BamHI to excise the 305
bp SmaI-I3L-FHV-1 gD 5'-most region-BamHI fragment. Plasmid
pJCA073 was digested with BamHI and XhoI to excise the 970
bp BamHI-FHV-1 gD 3'region-XhoI fragment. The SmaI-BamHI
305 bp and the BamHI-XhoI 970 bp fragments were finally
ligated together into vector pCPC6L (C6 deorfed locus; see

other ALVAC C6 donor plasmids for the generation of pCPC6L)
digested with SamI and XhoI. The plasmid resulting from
this ligation was designated pJCA084.

The plasmid pJCA084 was used in in vitro recombination
experiment in primary chick embryo fibroblasts with CPpp as
rescue virus to yield vCP162. Recombinant virus was
identified by in situ hybridization according to standard
procedures (Piccini et al., 1987) using a radiolabeled FHV-1
gD specific probe. Recombinant plaques were purified by
three rounds of plaque purification and amplified for
further analysis. Recombinant virus vCP162 contains the
FHV-1 gD coding sequence in the C6 locus of canarypox virus.

Example 58 - EXPRESSION ANALYSIS OF vCP162

Immunofluorescence assays were performed as described
previously (Taylor et al., 1990) using a sheep anti-FHV-1 gD
polyclonal serum obtained from D. Fargeaud, Rhone-Merieux,
France. Recombinant vCP162 displayed intense internal as
well surface fluorescence, indicating expression of FHV-1 gD
glycoprotein on the surface of the infected cells.

Immunoprecipitation was done using the same antiserum
to determine the authenticity of the expressed FHV-1 gD
product. This procedure was done according to that
previously described (Taylor et al., 1990). The sheep anti-
FHV-1 gD serum precipitated a product with an apparent
molecular mass on an SDS-PAGE gel system (Dreyfuss et al.,
1984) of 60 kDa from lysates derived from cells infected
with the recombinant virus vCP162. No protein was
precipitated from uninfected cells or lysates derived from
parental canarypox virus (CPpp) infected cells.

**Example 59 - GENERATION OF NYVAC- AND ALVAC-BASED
               RECOMBINANTS EXPRESSING THE HANTAAN VIRUS
               G1 AND G2 GLYCOPROTEINS**

Expression of the Hantaan virus G1 and G2 glycoproteins
was accomplished by insertion of the M segment into the
NYVAC and ALVAC vectors under the control of the
entomopoxvirus 42 kDa promoter. The poxvirus expression
cassette inserted into these viral vectors was constructed
as follows.

A cDNA clone of the Hantaan virus M segment was derived
as described by Schmaljohn et al. (1987) and provided by Dr.

J. Dalrymple (Virology Division, U.S. Army Medical Research
Institute of Infectious Disease, Frederick, MD) inserted in
the plasmid vector pTZ19R (Pharmacia, Piscataway, NJ). The
full sequence of the cDNA was presented previously by
Schmaljohn et al. (1987). The 326 bp 5'-most region of the
M segment coding sequence was derived using the plasmid
pTZ19R containing the M segment cDNA as template and
oligonucleotides HM5P (SEQ ID NO:335) (5'-ATGGGGA
TATGGAAGTGG-3') and HM3P (SEQ ID NO:336) (5'-CATGTT
CCTTTCAAGTCAAC-3'). This 326 bp fragment was fused to a 107
bp PCR-derived fragment containing the 42 kDa promoter
element obtained using pAM12 as template and
oligonucleotides RG273 (SEQ ID NO:142) (5'-AGGCAA
GCTTTCAAAAAAATATAAATGATTC-3') and RG274 (SEQ ID NO:143)
(5'-TTTATATTGTAATTATATATTTTC-3'). The PCR fusion was
performed using an equimolar mixture of the 107 bp and 326
bp fragments as template and oligonucleotides RG273(SEQ ID
NO:142) and HM3P(SEQ ID NO:336). The fused fragment was
digested with <u>Hin</u>dIII (5' end) and <u>Bcl</u>II (3' end) to yield a
343 bp fragment.
     The plasmid pAM12 was derived as follows. Genomic DNA
isolated from *Amsacta moorei* entomopoxvirus (AmEPV) was
digested with <u>Cla</u>I and the fragments separated by agarose
gel electrophoresis. A 2.6 kb fragment, previously
identified as containing a highly transcribed AmEPV gene,
was purified from the gel and cloned into the <u>Cla</u>I site of
pBS-SK+ yielding pAM12. DNA sequence analysis of this
fragment revealed a complete ORF of 42 kDa. The 5'
untranslated sequence from this gene (AmEPV 42K promoter)
was subsequently shown to function as a strong, early
promoter in both vaccinia and avipox viruses.
     The 3'-most 748 bp of the M segment coding sequence was
derived by PCR using the cDNA clone contained in pTZ19R as
template and oligonucleotides HMTS-5 (SEQ ID NO:337)
(5'-ATCATGAGTCCAAGAGACAAAGGTTTCTTATGCCCTGAG-3') and HMTS-3
(SEQ ID NO:338) (5'-ATCATCGAATTCATAAAAACTATGATTTTTTATGCTTCC
TTACGGGAC-3'). Synthesis of this fragment with these custom
synthesized oligonucleotides 1) contains the 3' most
sequences of and <u>Eco</u>RV site at the 5' end, 2) disrupts the

T$_5$NT sequence motif (Yuen and Moss, 1987) at nucleotide
positions 2687 to 2693 (Schmaljohn et al., 1987), and 3)
adds a T$_5$NT element and a unique EcoRI site at the 3' end of
the coding sequence. This fragment was digested with and
EcoRI to yield a 741 bp EcoRV/EcoRI fragment. The 741 bp
EcoRV/EcoRI fragment was inserted into pBS-SK (Stratagene,
La Jolla, CA) to yield plasmid pBSHVM3P.

The plasmid containing the M-specific cDNA clone in
pTZ19R was used to transform GM48 (Dam⁻) bacterial cells
(BRL, Gaithersburg, MD). Plasmid DNA derived from this
bacterial strain was digested with BclI and EcoRV to
generate a 2362 bp fragment. This 2362 bp fragment was co-
inserted into pBSHVM3P digested with HindIII and EcoRV with
the 343 bp HindIII/BclI fragment containing the 42 kDa
promoter fused to the 5' most region of the coding sequence.
The resultant plasmid containing the entire M segment
expression cassette was designated as pBSHVM. The entire M
segment cassette was excised from pBSHVM using restriction
endonucleases HindIII and EcoRI. The 3508 bp derived
fragment was blunt-end using the Klenow fragment of the *E.
coli* in the presence of 2mM dNTPs. The blunt ended fragment
was inserted into pSD550 to yield pHVMVC.

Plasmid pSD550 was derived from pSD548 as follows.
Plasmid pSD548 (Tartaglia et al., 1992) is a vaccinia vector
plasmid in which the I4L ORF (Goebel et al., 1990a, b) is
replaced by a cloning region consisting of BglII and SmaI
sites. To expand the multicloning region, pSD548 was cut
with BglII and SmaI and ligated with annealed complementary
synthetic oligonucleotides 539A (SEQ ID NO:339) (5'-AGAAAAA
TCAGTTAGCTAAGATCTCCCGGGCTCGAGGGTACCGGATCCTGATTAGTTAATTTT
TGT-3') and 539B (SEQ ID NO:340) (5'-GATCACAAAAATTAACTAAT
CAGGATCCGGTACCCTCGAGCCCGGGAGATCTTAGCTAACTGATTTTTCT-3'). In
the resulting plasmid, pSD550, the multicloning region
contains BglII, SmaI, XhoI, KpnI and BamHI restriction
sites.

The plasmid pHVMVC was used in *in vitro* recombination
experiments in Vero cells with vP804 (Tartaglia et al.,
1992) as rescue virus to yield vP882. Recombinant virus was
identified by *in situ* hybridization according to standard

procedures (Piccini et al., 1987) using a radiolabeled M-
specific DNA probe. Recombinant plaques were purified by 3
rounds of plaque purification and amplified for further
analysis. Recombinant virus, vP882, contains the Hantaan M
segment in the I4L locus of vaccinia virus. Replacement of
the I4L open reading frame with the M segment cassette in
the vP804 background creates a NYVAC- equivalent virus
background (Tartaglia et al., 1992).

    The 3508 bp HindIII/EcoRI fragment derived from pBSHVM,
containing the M segment cassette (above), was inserted into
pC4I digested with HindIII and EcoRI. The plasmid pC4I was
derived as follows. A 6.5 kb NsiI fragment from CPpp was
cloned into the BamHI site of pBS-SK+ yielding pXX-4. DNA
sequence analysis identified the complete open reading frame
(ORF) which contained the C4 insert in locus. In order to
construct a donor plasmid in which the C4 ORF is deleted,
oligonucleotides RG287 (SEQ ID NO:341) (5'-CAGTTGGTACC
TATGTTAAGGAGGACGA-3'), RG293 (SEQ ID NO:342) (5'-TATCTGAA
TTCCTGCAGCCCGGGTTTTTATAGCTAATTAGTCATATGATATTATCTCTAT-3'),
RG289 (SEQ ID NO:343) (5'-TCGATGAATTCGATATCAAGCTTATCGA
TTTTTATGATTAACTAGTCAAGTGATTTTATTCAATTACG-3'), and RG290 (SEQ
ID NO:344) (5'-TTATCGAGCTCATTTACATTTCTAAACTC-3') were used
as primers to amplify the 5' and 3' untranslated regions of
the C4 ORF from pXX4. The purified PCR fragments were
cloned into pBS-SK+ at the KpnI-SacI sites. The resulting
plasmid, pC4I, contains the 5' and 3' untranslated regions
of C4 (201 bp each) in which the complete ORF has been
replaced with a multiple cloning site flanked by
translational and vaccinia early transcriptional stop
signals (Yuen and Moss, 1987).

    Insertion of the M segment cassette into pC4I yielded
plasmid pC4HVM. The plasmid pC4HVM was linearized with SmaI
for insertion of a 100 bp SspI/SmaI fragment derived from
pSD513 (defined in Example 7). The resultant plasmid was
designated as pC4HVMVQ. Plasmid pC4HVMVQ was digested with
SmaI followed by a subsequent partial HindIII digestion to
recover a 3.6 kb fragment containing the M segment cassette.
This fragment was blunt-ended using the klenow fragment of
the E. coli DNA polymerase in the presence of 2mM dNTPs.

This blunt-ended fragment was inserted into SmaI digested
pSPCPC3L to generated pC3HVMVQ (defined in Example 32).

    In vitro recombination experiment were performed on
primary chick embryo fibroblasts using pC3HVMVQ as the donor
plasmid and CPpp (ALVAC) as the rescue virus. Standard
protocols were used to identify and to purify the
recombinant virus (as above; Piccini et al., 1987). The
ALVAC-based recombinant containing the Hantaan virus M
segment was designated as vCP114.

**Example 60 - GENERATION OF NYVAC- AND ALVAC-BASED
               RECOMBINANTS CONTAINING THE HANTAAN
               VIRUS S CODING SEQUENCE**

    Expression of the Hantaan virus nucleoprotein was
accomplished by insertion of the S segment into the NYVAC
and ALVAC vectors under the control of the vaccinia virus H6
promoter (Goebel et al., 1990a,b). The poxvirus expression
cassette inserted into these viral vectors was constructed
as follows.

    A cDNA clone of the Hantaan virus S segment was derived
as described by Schmaljohn et al. (1986) and provided by Dr.
Joel Dalrymple (Virology Division, U.S. Army Medical
Research Institute of Infectious Disease, Ft. Detrick,
Frederick, MD) inserted into the plasmid vector pGEM-1
(Promega Biotech, Madison, WI). The entire sequence of the
S segment has been described previously (Schmaljohn et al.,
1986).

    The H6 promoter element was amplified by PCR using
plasmid pI4LH6HIV3B as template and oligonucleotides
H65PH(SEQ ID NO:164) (5'-ATCATCAAGCTT-3') and HTH63P (SEQ ID
NO:346) (5'-CCCTCTGTAATTCCTCCATAGTTGCCATTACGATACAAACTT
AACGG-3'). This 150 bp PCR-derived fragment contains a
HindIII site at the 5'-end and has a 28 bp extension at the
3' end containing the 5'-most region of the S segment coding
sequence.

    The 5' end of the S segment coding sequence was
synthesized by PCR with oligonucleotides HTS5P (SEQ ID
NO:347) (5'-CCGTTAAGTTTGTATCGTAATGGCAACTATGGAGG-3') and
HTS5PNCI (SEQ ID NO:348) (5'-GGACACGTAAAGATGTTTTGG-3') and
the S-specific cDNA clone as template. The 560 bp PCR-
derived fragment was fused by PCR using oligonucleotides

H65PH (SEQ ID NO:164) and HTS5PNCI (SEQ ID NO:348). The
PCR-derived fusion product was digested with _Hin_dIII and
_Nci_I.

The central region of the Hantaan virus S segment was
generated by PCR using oligonucleotides T5HT3PPS (SEQ ID
NO:349) (5'-GTCCTGCAGGATGGAAAAGAATGCCCCAAGC-3') and HTS55PN
(SEQ ID NO:350) (5'-GGGGGAGGCAAACTACCAAGG-3') and the S-
specific cDNA clone as template. The 581 bp fragment
contains a _Pst_I site at its 3' end and the 5' end includes
the _Nci_I site of position 499 of the S segment (Schmaljohn
et al., 1986). Furthermore, using the oligonucleotide
T5HT3PPS (SEQ ID NO:349) eliminates the T_5NT element at
position 1029 to 1035 without altering the amino acid
sequence. This fragment was then digested with _Nci_I and
_Pst_I. The PCR fragment containing the 5' end of the coding
sequence fused to the H6 promoter (_Hin_dIII/_Nci_I digested
above) was ligated into pBS-SK digested with _Hin_dIII and
_Pst_I along with the 581 bp _Nci_I/_Pst_I fragment containing the
central region of the S segment. The resultant plasmid was
designated pBSHTSH65P.

The 3' most 438 bp of the S segment was derived by PCR
using oligonucleotides HTS3PXBA (SEQ ID NO:351) (5'-ATCATC
TCTAGAATAAAAATTAGAGTTTCAAAGGC-3') and T5HT5PSP (SEQ ID
NO:352) (5'-CGCCAGCATGCAGAAGCAGC-3') and the S-specific cDNA
clone as template. The 5' end of this fragment contains the
_Pst_I site situated at position 1039 of the S segment coding
sequence (Schmaljohn et al., 1986) and the 3' end contains a
T_5NT sequence motif and a unique _Xba_ I site. This fragment
was digested with _Pst_I and _Xba_I prior to insertion into
_Pst_I/_Xba_I digested pBS-SK to yield pBSHTS3P.

To generate the entire S segment expression cassette, a
1122 bp _Pst_I/partial _Hin_dIII fragment was derived from
pBSHTSH65P. This fragment was co-inserted into _Hin_dIII/_Xba_I
digested pBS-SK with a 290 bp _Pst_I/_Xba_I fragment from
pBSHTS3P. The resultant plasmid was designated pBSHVS by
linearization with _Xba_I followed by a partial _Hin_dIII
digestion. This fragment was blunt-ended using the klenow
fragment of the _E. coli_ DNA polymerase in the presence of
2mM dNTPs and then inserted into the _Sma_I site of pSD541

(defined in Example 10) to generate pATIHVS.

The plasmid pATIHVS was used in *in vitro* recombination experiments in Vero cells with vP866 (NYVAC) and vP882 as rescue virus. This was performed with standard protocols (Piccini et al., 1987). Plaques derived from recombinant virus were identified by a plaque hybridization procedure defined previously (Piccini et al., 1987) using a radiolabeled S segment-specific DNA probe. The generated recombinants were designated as vP950 and vP951 for virus recovered using the rescue virus vP866 and vP882, respectively. Recombinant virus, vP950, contains the S segment expression cassette in the ATI site and vP951 contains this cassette at the same locus, but by virtue of rescue with the M segment containing vP882, also contains the M segment in the I4L locus.

The plasmid pBSHVM was linearized with SalI and blunt-ended using the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2mM dNTPs. This was ligated to the 1.4 kb XbaI/partial HindIII (Blunt-ended with Klenow as above) fragment derived from pBSHVS containing the Hantaan S segment expression cassette. The derived plasmid was designated as pBSHVMS. This plasmid contained the M and S cassettes in a head to head configuration. Plasmid pBSHVMS was linearized with XhoI, blunted with Klenow (as above), and ligated to a 100 bp SspI/SmaI fragment from pSD513 (defined in Example 7) to yield pBSHVMSVQ.

The plasmid pBSHVMSVQ was digested with ClaI/Asp718 to liberate a 1.5 kb fragment containing the S segment expression cassette. This fragment was blunt-ended using the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2mM dNTPs and inserted into the SmaI site of pSPCP3L (defined in Example 32) to yield pC3HVSVQ.

Plasmid pC3HVSVQ was used in *in vitro* recombination experiments in primary chick embryo fibroblasts with CPpp (ALVAC) as the rescuing virus. This was performed using standard procedures, as were the plaque identification, plaque purification, and virus amplification (Piccini et al., 1987). The recombinant generated was designated as vCP119. This recombinant contains the S segment in the C3

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locus of ALVAC.

**Example 61 - EXPRESSION ANALYSIS OF THE NYVAC-
                AND ALVAC- BASED HANTAAN VIRUS
                M AND S SEGMENT RECOMBINANTS**

     Immunofluorescence assays were performed as described
previously (Taylor et al., 1990) using either a pool of
monoclonal antibodies specific to G1, G2, or a rabbit anti-
Hantaan polyclonal serum. All sera were obtained from Dr.
C. Schmaljohn (Virology Division, U.S. Army Medical Research
Institute of Infectious Diseases, Ft. Detrick, Frederick,
MD). The recombinant viruses containing the M segment alone
(vP882, and vCP114) or in combination with the S segment
(vP951) displayed intense surface fluorescence using any of
the above antiserum, indicating expression of both the G1
and G2 glycoproteins on the surface of the infected cells.
Surface fluorescence was not observed using the recombinants
containing the S segment alone (vP950 and vCP119), since the
nucleoprotein is not displayed on the surface of infected
cells. Internal fluorescence was observed, however, with
the recombinants, vP950 and vCP119, using the rabbit anti-
Hantaan serum.

     Immunoprecipitations were done using the same anti-sera
to determine the authenticity of the expressed G1, G2, and S
gene products. This procedure was done according to that
previously described (Taylor et al., 1990). Monoclonal
antibody pools specific for G1 precipitated a protein with
an apparent molecular mass on an SDS-PAGE gel system
(Dreyfuss et al., 1984) of 64 kDa from lysates derived from
cells infected with the recombinant viruses vP882, vP951,
and vCP114. No protein was precipitated from uninfected
cells or lysates derived from either parental (NYVAC and
ALVAC) infected cells or cells infected with the recombinant
viruses containing only the S segment (vP950 and vCP119).
Equivalent results were obtained using the monoclonal
antibody pool specific for G2, except that the polypeptide
precipitated had a molecular mass of approximately 50-55
kDa. These size proteins (64 kDa and 50-55 kDa) are
consistent with the size of the G1 and G2 proteins,
respectively (Schmaljohn et al., 1990).

     The rabbit anti-Hantaan precipitated three protein

species with apparent molecular weights of 64 kDa 50-55 kDa,
and 48 kDa from lysates derived from cells infected with
vP951, consistent with expression of G1, G2 and the
nucleoprotein. The same serum specifically precipitated the
G1 and G2 (64 kDa, 50-55 kDa) species from vP882 and vCP114
and the nucleoprotein (48 kDa) from vP950 and vCP119
infected cell lysates. No such proteins were precipitated
from lysates derived form uninfected cells or cells infected
with the NYVAC or ALVAC parent viruses.

**Example 62 - INSERTION OF HEPATITIS B VIRUS (HBV) GENES
                 INTO AN ALVAC INSERTION PLASMID**

ALVAC C5 insertion plasmid pNVQH6C5LSP18 (defined in
Example 44) was cut with BamHI and ligated with kinased,
annealed synthetic oligonucleotides MPSYN438/MPSYN439 (SEQ
ID NOS:353/354).
MPSYN438 5' GATCCCCGGGA 3'
MPSYN439 5' GATCTCCCGGG 3'
The resulting plasmid, pMPC5Sma, contains a SmaI site next
to the BamHI site in the linker region.

pMP550E31lps (defined in other HBV example herein) was
cut with BglII/BamHI (partial) and a 1.2 kb fragment
containing the HBV lpsAg gene under the control of the EPV
42kDa promoter was isolated and ligated with pMPC5Sma cut
with BamHI, resulting in plasmid pMPC5L. pMPC5L was cut
with SmaI (partial) and the unit length (4 kb) fragment was
isolated. This fragment was cut with NruI and the largest
band (4 kb) isolated.

pGJ15 (defined in Example 13) was digested with SmaI
(partial) and NruI and a 0.9 kb fragment, containing the HBV
spsAg and 24 bp of the H6 promoter, was isolated and ligated
to the 4 kb SmaI/NruI vector fragment from pMPC5L,
generating plasmid pMPC5LS.

pMPI3S12C (defined in Example 13) was cut with SmaI,
and a 1.2 kb fragment containing the S12/core fusion gene
under the control of the I3L promoter was isolated. This
fragment was ligated into plasmid pMPC5LS which had been cut
with SmaI, resulting in plasmid pMPC5LSC.

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**Example 63   - INSERTION OF HBV GENES ENCODING SPSAG
                   AND LPSAG INTO ALVAC**

pMPC5LS, containing both the HBV lpsAg and spsAg in the
C5 insertion locus, was used as donor plasmid for
recombination with ALVAC, generating ALVAC recombinant
vCP157.

Expression of HBV proteins by vCP157. Metabolically
labelled lysates from cells infected with ALVAC-HBV double
recombinant vCP157 were subjected to immunoprecipitation
using rabbit anti-S2 serum, and analyzed by SDS-
polyacrylamide gel electrophoresis followed by
radioautography. Proteins of the correct size for lpsAg (41
kDa, 38 kDa) and spsAg (36 kDa, 33 kDa), were observed.
These proteins co-migrated with the same proteins produced
by NYVAC single HBV recombinants expressing spsAg or lpsAg
which were used as controls.

**Example 64   - INSERTION OF HBV GENES ENCODING SPSAG,
                   LPSAG AND THE S12/CORE FUSION INTO ALVAC**

pMPC5LSC, containing both the HBV lpsAg and spsAg genes
as well as the gene specifying the S12/core fusion in the C5
insertion locus, was used as donor plasmid for recombination
with ALVAC, generating ALVAC recombinant vCP169.

**Example 65   - NYVAC-BASED RECOMBINANTS EXPRESSING THE HBV
                   GENES: SEROLOGICAL DATA FROM RABBITS, MICE
                   AND GUINEA PIGS**

Rabbits, mice and guinea pigs were inoculated with
NYVAC-based Hepatitis B virus (HBV) recombinants vP856,
vP930, vP932 and vP975 (Example 13). vP856 expresses spsAg,
the middle (M) form of the surface antigen. vP930 expresses
lpsAg, the large (L) form of the surface antigen. vP932
expresses both spsAg and lpsAg. vP975 expresses spsAg,
lpsAg and a fusion protein composed of the surface antigen
preS1 + preS2 regions fused to the core antigen. Sera were
analyzed for antibody to HBV surface antigen using the AUSAB
test (Abbott), and for antibody to HBV core antigen using
the CORAB competitive radioimmunoassay kit (Abbott).
Antibody to HBV preS1 and preS2 regions were assayed by
ELISA. Results are presented in Tables 34-40.

Table 34.   AUSAB and CORAB

Rabbits

Analysis of sera of rabbits inoculated with NYVAC-based HBV recombinants expressing the middle (M) form of the surface antigen (small pre S antigen), the large (L) form of the surface antigen (large pre S antigen) and a fusion protein (S/C) consisting of the pre S 1+2 regions fused to the core antigen.

AUSAB[a]

#	vP	HBV genes	1	2	3	4	5	6	7	8
							week			
A133	932	M + L	>512	>512	262	352	13000	6500	3600	5400
A134	932	M + L	250	235	72	80	3900	561	800	218
A135	975	M + L + S/C	36	58	274	406	1300	646	436	268
A136	975	M + L + S/C	103	>512	127	136	13468	4968	3168	2768

CORAB[b]

#	vP	HBV genes	1	2	3	4	5	6	7	8
							week			
A135	975	M + L + S/C	80	20	20	80	320	80	320	80
A136	975	M + L + S/C	20	20	5	5	80	80	320	80

Rabbits were inoculated with 10^8 pfu of the indicated vaccinia recombinant at week 0 by the IM route, and boosted by the same route and dose at week 4.

[a] Sera were analyzed by AUSAB radioimmunoassay kit (Abbott). Titers are expressed in RIA units. Note: Three sera (indicated ">512") scored above the sensitivity of the AUSAB test for undiluted sera (512 RIA units) and below the sensitivity of the test for sera diluted 1:100 (800 RIA units).

Table 35. Pre-S2 ELISA

Analysis by ELISA of sera from rabbits inoculated with NYVAC-based HBV recombinants expressing the middle (M) form of the surface antigen, the large (L) form of the surface antigen and a fusion protein (S/C) consisting of the pre S 1+2 regions fused to the core antigen.

Rabbits

#	vP	HBV genes	week							
			1	2	3	4	5	6	7	8
A133	932	M + L	0	0	29	35	474	602	358	419
A134	932	M + L	0	0	0	277	2017	3099	847	500
A135	975	M + L + S/C	0	0	0	0	175	105	94	48
A136	975	M + L + S/C	0	0	0	0	2440	763	672	355

Rabbits were inoculated by the intramuscular (IM) route with 10^8 pfu of the indicated vaccinia recombinant at week 0 and boosted by the same route and dose at week 4.

Sera were analyzed by indirect ELISA for the detection of antibodies to HBV pre-S2 region (aa 120-145). Microtiter plates were coated with HBV pre-S2 region peptide (adw 120-145) at a concentration of 50 ng/well. Each serum sample was assayed in duplicate, with initial dilution of 1:10 followed by two-fold serial dilutions. Numbers, based on a standard curve, reflect the dilution factor giving an optical density of 0.25 (compared to 0.14 for prebleed).

Table 36.   Pre-S1 ELISA

Analysis by ELISA test of sera from rabbits inoculated with NYVAC-based
HBV recombinants expressing the middle (M) form and the large (L) form
of the surface antigen and the preS1+2/core fusion protein (S/C).

Rabbits

#	vP	HBV genes	week							
			0	2	4	5	6	8		
A133	932	M + L	<10	<10	<10	<10	<10	<10		
A134	932	M + L	<10	<10	<10	<10	17	<10		
A135	975	M + L + S/C	<10	<10	15	40	<10	24		
A136	975	M + L + S/C	15	16	17	117	52	49		

Rabbits were inoculated by the intradermal (ID) route with 10^8 pfu of
the indicated vaccinia recombinant at week 0 and boosted by the same
route and dose at week 4.

Sera were analyzed by indirect ELISA for the detection of antibodies to
HBV pre-S1 region. Microtiter plates were coated with HBV pre-S1
peptide (subtype adw aa 20-54, obtained from Dr. Colin Howard, The
Royal Veterinary College, London) at a concentration of 500 ng/well.
Each serum sample was assayed in duplicate, with initial dilution of
1:10 followed by two-fold serial dilutions. Numbers, based on a
standard curve, reflect the dilution factor giving an optical density
of 0.20.

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Table 37.　Pre-S2 ELISA

Guinea Pigs

Analysis by ELISA of sera from guinea pigs inoculated with NYVAC-based HBV recombinants expressing the middle (M) form of the surface antigen, the large (L) form of the surface antigen and the preS1+2/core fusion protein (S/C).

#	vP	HBV genes	week			
			0	5	6	
85	856	M	<10	<10	<10	
86	856	M	<10	<10	<10	
87	930	L	<10	46	35	
88	930	L	<10	30	93	
89	932	M+L	<10	39	<10	
90	932	M+L	<10	33	19	
91	975	M+L+S/C	<10	22	84	
92	975	M+L+S/C	<10	53	269	

Guinea pigs were inoculated by the SC route with 10^8 of the indicated HBV recombinant vaccinia virus. Animals were boosted at 4 weeks with the same dose and route. Sera were analyzed by indirect ELISA for the detection of antibodies to HBV preS2 region. Microtiter plates were coated with synthetic S2 peptide (subtype adw, aa 120-145, Bachem Bioscience) at a concentration of 50ng/well. Each serum sample was assayed in duplicate with initial dilution of 1:110 followed by two-fold serial dilutions. Titers, based on standard curve, represent the dilution factor giving an optical density of 0.45 (compared to a mean prebleed value of 0.26).

Table 38. CORAB

Analysis of sera by CORAB test of mice inoculated with vaccinia recombinant vP975 expressing the HBV middle (M) form of the surface antigen, the large (L) form of the surface antigen and a fusion protein (S/C) consisting of the pre S 1 + 2 regions fused to the core antigen.

Mice

Group	vP	HBV genes		Week						
			1	2	3	4	5	6	7	8
D	975	M+L+S/C	-ᵃ	-	-	-	5	5	5	5

Mice were inoculated by the IM route with 10⁷ pfu of vP975 and boosted at 4 weeks with the same dose and route. A total of 12 mice were inoculated. Sera from each weekly bleeding were pooled.

Sera diluted 1:5, 1:20, 1:80 or 1:320 with bovine serum were analyzed by CORAB competitive radioimmunoassay kit (Abbott). The reciprocal of the highest dilution giving a positive score is indicated. In all cases, the sample was tested at at least one further dilution beyond the value indicated.

ᵃ"-" indicates that mouse sera diluted 1:5 with bovine serum gave a negative score.

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Table 39. Pre-S2 ELISA

Analysis by ELISA of sera from mice inoculated with NYVAC-based HBV recombinants expressing the middle (M) form of the surface antigen, the large (L) form of the surface antigen and the preS1+2/core fusion protein (S/C).

			week		
	vP	HBV genes	0	5	6
Group A	856	M	<10	73	70
Group B	930	L	<10	93	112
Group C	932	M+L	<10	970	1146
Group D	975	M+L+S/C	<10	1054	1062

Groups of eight or twelve mice were inoculated by the IM route with 107 of the indicated HBV recombinant vaccinia virus. Animals were boosted at four weeks with the same dose and route. Sera from each group for each bleeding were pooled.

Sera were analyzed by indirect ELISA for the detection of antibodies to HBV preS2 region. Microtiter plates were coated with synthetic S2 peptide (subtype adw, aa 120-145, Bachem Bioscience) at a concentration of 50ng/well. Each serum sample was assayed in duplicate, with initial dilution of 1:10 followed by two-fold serial dilutions. Titers based on a standard curve, represent the dilution factor giving an optical density of 0.25 (compared to a mean prebleed value of 0.11).

Table 40. Pre-S1 ELISA

Analysis by ELISA of sera from mice inoculated with NYVAC-based HBV recombinants expressing the middle (M) form of the surface antigen, the large (L) form of the surface antigen and the preS1+2/core fusion protein (S/C).

Mice

			week	
	vP	HBV genes	0	5
Group B	930	L	60	244
Group C	932	M+L	66	125
Group D	975	M+L+S/C	63	1554

Groups of eight or twelve mice were inoculated by the IM route with 10^7 of the indicated HBV recombinant vaccinia virus. Animals were boosted at 4 weeks with the same dose and route. Sera from each group for each bleeding were pooled.

Sera were analyzed by indirect ELISA for the detection of antibodies to HBV preS1 region. Microtiter plates were coated with synthetic S1 peptide (subtype adw, aa 20-54, obtained from Dr. Colin Howard, The Royal Veterinary College, London) at a concentration of 500ng/well. Each serum sample was assayed in duplicate, with initial dilution of 1:10 followed by two-fold serial dilutions. Titers, based on a standard curve, represent the dilution factor giving an optical density of 0.2.

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Example 66   -   EXPRESSION OF BACTERIAL GENES IN POXVIRUS:
                  TETANUS TOXIN FRAGMENT C (C. TETANI)
                  IN POXVIRUSES

C. tetani is bacteria of the genus *clostridium*, spore
forming anaerobes; included in the genus is also *C.
botulinum*. *C. Tetani* produces a toxin, tetanospasmin (TT)
which is primarily responsible for the paralytic effects
observed upon infection. TT is a single 150 kDa light chain
and 100 kDa heavy chain upon release from the bacteria. The
heavy chain yields two fragments, B and C (45 kDa and 55
kDa) upon mild proteolytic treatment. This Example
demonstrates that viruses of the invention can be used to
express immunogenic bacterial gene products such as fragment
C of *C. tetani*.

The expression cassette was generated by a series of
polymerase chain reactions. This approach was necessary to
remove a TTTTTAT early transcription termination signal
(Yuen and Moss, 1987) present in the native tetanus toxin
sequence (Eisel et al. ,1986; Fairweather and Lyness, 1986).

The H6 promoter-linked fragment C was derived by PCR
from plasmid pSS1261 (Halpern et al., 1990) using primers
H6TETC (SEQ ID NO:355) (5'- ATCATCGCGATATCCGTTAAGTTTGTATCG
TAATGAAAAATCTGGATTGTTGGG -3') and TETFIX2 (SEQ ID NO:356)
(5'- GTAATAGTTATGAAAACCC -3'). The remainder of the coding
region for the construct was generated by PCR amplification
of plasmid pSS1261 using primers TETFIX1 (SEQ ID NO:357)
(5'- GGGTTTTCATAACTATTAC -3') and TETEND (SEQ ID NO:358)
(5'-GGATGGACAAATGATTAATTTTTATCTCGAGCCCGGGATGAT -3'). Fusion
of these coding regions was accomplished by PCR
amplification of these 553 bp and 881 bp PCR-derived
products using primers H6TETC (SEQ ID NO:355) and TETEND
(SEQ ID NO:358). The resulting 1.4 kb PCR-derived product
was digested with EcoRV and XhoI. A fragment of 1.4 kb was
isolated from an agarose gel and ligated to similarly
digested pBS-SK+ (Stratagene, La Jolla, CA). Nucleotide
sequence analysis was used to verify the insert in the
resulting plasmid pBSTETC. A T to C silent substitution was
found in this plasmid, corresponding to position 3535 in the
sequence of Eisel et al. (1986).

pC5L (defined in Example 44) was digested within the

polylinker with Asp718 and NotI, treated with alkaline
phosphatase and ligated to kinased and annealed
oligonucleotides CP26 (SEQ ID NO:359)
(5'- GTACGTGACTAATTAGCTATAAAAAGGATCCGGTACCCTCGAGTCTAGAATCGAT
CCCGGGTTTTTATGACTAGTTAATCAC -3') and CP27 (SEQ ID NO:360)
(5'- GGCCGTGATTAACTAGTCATAAAAACCCGGGATCGATTCTAGACTCGAGGGTACC
GGATCCTTTTTATAGCTAATTAGTCAC -3') (containing a disabled
Asp718 site, translation stop codons in six reading frames,
vaccinia early transcription termination signal (Yuen and
Moss, 1987), BamHI, KpnI, XhoI, XbaI, ClaI, and SmaI
restriction sites, vaccinia early transcription termination
signal, translation stop codons in six reading frames, and a
disabled NotI site) generating plasmid pC5LSP. The
early/late H6 vaccinia virus promoter (Perkus et al., 1989)
was derived by PCR from a plasmid containing the promoter
using oligonucleotides CP30 (SEQ ID NO:361)
(5'- TCGGGATCCGGGTTAATTAATTAGTCATCAGGCAGGGCG -3') and CP31
(SEQ ID NO:362) (5'- TAGCTCGAGGGTACCTACGATACAAACTTAACG
GATATCG -3'). The PCR product was digested with BamHI and
XhoI (sites created at the 5' and 3' termini by the PCR) and
ligated to similarly digested pC5LSP generating pVQH6C5LSP.
pVQH6C5LSP was digested with EcoRI, treated with alkaline
phosphatase, ligated to self-annealed oligonucleotide CP29
(SEQ ID NO:363) (5'- AATTGCGGCCGC -3'), digested with NotI
and linear purified followed by self-ligation. This
procedure introduced a NotI site to pVQH6C5LSP, generating
pNVQH6C5LSP18. The 1.4 kb EcoRV/XhoI fragment from pBSTETC
was isolated and ligated to similarly digested pNVQH6C5LSP18
creating pC5TETC.

     The H6 promoter through the EcoRV site was derived from
a plasmid containing the synthetic H6 promoter (Perkus et
al., 1989), using PCR and primers H6PCR1 (SEQ ID NO:364)
(5'- ACTACTAAGCTTCTTTATTCTATACTTAAAAAGTG -3') and H6PCR2
(SEQ ID NO:365) (5'- TTAACGGATATCGCGATAATG -3'), creating a
5' HindIII site. This 122 bp PCR-derived fragment was
digested with HindIII and EcoRV followed by ligation to
similarly digested pBS-SK+ (Stratagene, La Jolla, CA),
generating plasmid pBSH6. The insert was confirmed by
nucleotide sequence analysis. The 1.4 kb EcoRV/XbaI

fragment from pC5TETC was excised and ligated to similarly
digested pBSH6, creating pH6TETC.

A 1.5 kb XhoI fragment from pH6TETC containing the
entire H6-Fragment C cassette was then ligated to pSD542
(defined in Example 32) creating pTKTETC.

pC5TETC and pTKTETC were employed in in vitro
recombination experiments with ALVAC and NYVAC creating
recombinants vCP161 and vP1075, respectively.

Immunoprecipitation analysis was performed to
determmine whether vCP161 and vP1075 express authentic
fragment C. Vero cell monolayers were either infected with
parental virus, CPpp (ALVAC) or vP866 (NYVAC), or infected
with vCP1661 or vP1075 at an m.o.i. of 10 pfu/cell. Cells
were infected, incubated in modified Eagle's medium (minus
methionine) containing [^{35}S]-methionine (20 μCi/ml), lysed
and precipitated as described by Taylor et al. (1990) using
a mouse monoclonal antibody (clone 49.4, Boehringer
Mannheim, Indianapolis, IN) and goat anti-mouse antisera as
secondary antibody. Lysates were precleared with normal
mouse sera and protein A-sepharose.

The monoclonal antibody specifically precipitated a
species corresponding to approximately 47 kDa from vCP161
and vP1075 infected cells. No similar protein species were
precipitated from either CPpp (ALVAC) or vP866 (NYVAC)
infected cells. The 47 kDa size is in agreement with
observations by Makoff et al., 1989) for fragment C produced
by papain digestion of native tetanus toxin as well as an E.
coli produced recombinant fragment C which is identical to
that encoded by vCP161 and vP1075.

**Example 67 - EFFICACY OF NYVAC-BASED PSEUDORABIES VIRUS
(PRV) RECOMBINANTS IN SWINE**

Six groups of four piglets were inoculated with either
the NYVAC-based gII recombinant (vP881), gIII recombinant
(vP883), gp50 recombinant (vP900), the NYVAC parental virus
(vP866), a commercially available inactivated pseudorabies
virus vaccine, or Eagle's MEM containing fetal bovine serum
as a sham vaccine. All pigs received two inoculations 28
days apart via the intramuscular route. The pigs receiving
parental or recombinant NYVAC viruses were inoculated with

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approximately 10^6 TCID$_{50}$ per inoculation. All pigs were monitored for body temperature and clinical signs for 12 days post-inoculation. No local or systemic adverse reactions were observed to vaccination with recombinant or parental NYVAC viruses.

Serum neutralizing antibody titers against pseudorabies virus were monitored for 1, 2, and 4 weeks following each inoculation. As is evident from Table 41, only the inactivated vaccine preparation was able to elicit significant pseudorabies virus serum neutralizing titers following a single inoculation. However, following a second inoculation all three NYVAC-based recombinants were able to elicit observable pseudorabies virus serum neutralizing titers (Table 42). The titers were most significant with the NYVAC recombinant expressing the pseudorabies virus gp50 glycoprotein. In fact, the titers elicited by the gp50 expressing NYVAC recombinant were comparable to levels attained with two inoculations of the commercially available inactivated vaccine (Table 42).

Four weeks following the second immunization all pigs were challenged with approximately 10^5 PFU of a virulent pseudorabies virus strain via intranasal instillation. Expectedly, the sham and parental NYVAC vaccinated controls all demonstrated the most severe clinical symptoms and the highest levels of pseudorabies virus isolated via nasal and oropharyngeal swabs up to 14 days post-challenge. NYVAC-based pseudorabies virus recombinant viruses were all shown to reduce the effects of the virulent pseudorabies virus challenge (i.e. clinical signs and virus isolation) compared to the controls, with the gp50 expressing recombinant virus being the most efficacious. In fact, this recombinant was as effective as the commercially available inactivated pseudorabies virus vaccine.

Since PRV, like other herpesviruses, has the potential of developing a latent infection that can be reactivated by stress or corticosteroid administration (Whittmann and Rziha, 1989), experiments were performed with two pigs were group to evaluate protective efficacy of the NYVAC recombinants against such infections. The protocol

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involved administering dexamethasone to the pigs in the following schedule: 1.25 mg/pound via the intravenous route and 0.25 mg/pound via the intravenous route on day one. This was followed by subsequent dexamethasone administration (0.5mg/pound) approximately every 12 hr for four days. Virus shedding was evaluated from day 0 to day 10 post-dexamethasone treatment using nasal (N) and throat (T) swabs. The results are shown in Table 43. These results clearly demonstrate the efficacy of the NYVAC-based PRV recombinant viruses in protecting against the establishment of latent PRV infections. The NYVAC-gII (vP881) was indeed the most efficacious by this criterion.

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Table 41.

PRV SN Titers Following First Immunization.

Pig	Group	Day 0	1 week	2 weeks	4 weeks
1		<2	<2	<2	<2
2	NYVAC/gII	<2	<2	<2	<2
3		<2	<2	<2	<2
4		<2	<2	<2	<2
5		<2	<2	<2	2
6	NYVAC/gIII	<2	<2	<2	2
7		<2	<2	<2	2
8		<2	<2	<2	2
9		<2	<2	8	<2
10	NYVAC/gp50	<2	<2	4	8
11		<2	2	2	.4
12		<2	2	2	8
13		<2	<2	<2	<2
14	NYVAC	<2	<2	<2	<2
15		<2	<2	<2	<2
16		<2	<2	<2	<2
17		<2	8	128	64
18	Inact.	<2	16	512	256
19	vaccine	<2	16	128	128
20		<2	2	16	8
21		<2	<2	<2	<2
22	Control	<2	<2	<2	<2
23		<2	<2	<2	<2
24		<2	<2	<2	<2

Table 42. PRV SN Titers Following Second Immunization

Pig	Group	1 week	2 weeks	4 weeks*
1		16	32	16
2	NYVAC/gII	32	16	8
3		64	64	8
4		128	64	64
5		16	32	8
6	NYVAC/gIII	8	8	8
7		8	8	4
8		16	64	16
9		2048	>4096	1024
10	NYVAC/gp50	>4096	4096	2048
11		1024	1024	512
12		4096	2048	1024
13		<2	<2	<2
14	NYVAC	<2	<2	<2
15		<2	<2	<2
16		<2	<2	<2
17		>4096	>4096	2048
18	Inact. vaccine	>4096	>4096	4096
19		2048	2048	256
20		>4096	>4096	2048
21		<2	<2	<2
22	Control	<2	<2	<2
23		<2	<2	<2
24		<2	<2	<2

Table 43 - VIRUS ISOLATION POST DEXAMETHASONE

Const	Pig	Site	d-0	d-1	d-2	d-3	d-4	d-5	d-6	d-7	d-8	d-9	d-10
gII	3	N	-	-	-	-	-	-	-	-	-	-	-
		T	-	-	-	-	-	-	-	-	-	-	-
	4	N	-	-	-	-	-	-	-	-	-	-	-
		T	-	-	-	-	1+	3+	(+)	-	-	-	-
gIII	6	N	-	-	-	(+)	1+	-	-	-	-	-	-
		T	-	-	(+)	1+	1+	-	-	-	-	-	-
	7	N	-	-	1+	(+)	-	1+	-	-	-	-	-
		T	-	-	1+	2+	3+	-	-	-	-	-	-
gp50	9	N	-	-	-	-	-	-	-	-	(+)	-	-
		T	-	-	-	-	-	1+	(+)	1+	1+	(+)	(+)
	11	N	-	-	-	-	-	(+)	3+	(+)	(+)	-	-
		T	-	-	(+)	(+)	-	4+	1+	1+	-	-	-
parent vac	14	N	-	-	2+	4+	4+	4+	-	-	-	-	-
		T	-	-	(+)	2+	2+	3+	-	-	-	-	-
	16	N	-	-	1+	2+	-	(+)	-	-	-	-	-
		T	-	-	-	1+	-	-	-	-	-	-	-

Table 43   -   (continued)

Const	Pig	Site	d-0	d-1	d-2	d-3	d-4	d-5	d-6	d-7	d-8	d-9	d-10
inact PRV	17	N	-	-	1+	(+)	1+	-	-	-	-	-	-
		T	-	-	-	-	-	-	-	-	-	-	-
	19	N	-	-	-	-	-	-	-	-	-	-	-
		T	-	-	1+	1+	1+	(+)	-	(+)	-	-	-
cont	22	N	-	-	1+	-	-	-	-	-	-	-	-
		T	-	-	-	-	-	-	-	-	-	-	-
	24	N	-	-	3+	2+	2+	3+	1+	-	-	-	-
		T	-	-	2+	2+	1+	2+	2+	1+	-	-	-

1+ = 1-10 plaques @ 24 hr;

2+ = 11-100 plaques @ 24 hr;

3+ = 101-1000 plaques @ 24 hr;

4+ = complete CPE @ 24 hr;

(+) = plaques after 24 hr.

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Example 68 - USE OF NYVAC.1 (vP954) AS A VECTOR

In NYVAC.1 (vP954) (Example 17) the [C7L-K1L] deletion in NYVAC (vP866) is expanded to the right, through K3L, a gene whose deletion confers heightened sensitivity to interferon (Beattie et al., 1991). NYVAC.1 was tested as an immunization vector for the rabies glycoprotein and for the measles HA and F genes as detailed below.

Insertion of the Rabies Glycoprotein Gene Into vP954: Generation of Vaccinia Recombinant vP1006. Donor plasmid pRW842 (Example 7), which contains an expression cassette for the rabies glycoprotein inserted in the TK deletion locus, was used in recombination with vP954 rescuing virus. Recombinant vaccinia virus vP1006 was identified by plaque hybridization using ^{32}P- labelled DNA probe to rabies glycoprotein G coding sequences.

Insertion of Genes Encoding Measles HA, F Into vP954: Generation of Vaccinia Recombinant vP1015. pRW857 (Example 9), which contains expression cassettes for both the measles F and measles HA in the TK insertion locus, was used as donor plasmid for recombination with vP954 rescuing virus. Recombinant virus vP1015 was identified by plaque hybridization using ^{32}P-labelled probe to both the measles HA and the measles F coding sequences.

Efficacy of K3L-MINUS NYVAC.1 Based Vector As An Immunization Vehicle: Rabies G recombinant vP1006. Four to six week old mice (10 per dilution per virus) were inoculated with either undiluted 10^{-2}, 10^{-4}, or 10^{-6} dilutions of vP1006, vP954 (parent of vP1006), vP879 (NYVAC-based rabies G, Example 7), or vaccinia rabies 187 XP1^2. The inoculations were performed in 50-100μl per mouse via the footpad. Fourteen days post-inoculation all mice were challenged with an intercerebral inoculation of 20 LD$_{50}$ of the CVS strain (in 0.03ml). Surviving mice were counted and protective dose 50% (PD$_{50}$) was calculated.

As shown in Table 44, the parent virus of vP1006, vP954, did not protect any mice from live rabies challenge even at the highest inoculum. Recombinant viruses 187 XP12 (Kieny et al., 1984), vP879, and vP1006 all demonstrated the ability to protect mice from live rabies challenge. The

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relative efficacy of these viruses in this capacity did
however vary as illustrated by the PD_{50} values (Table 44).

    <u>Measles Virus Recombinant vP1015</u>. Two rabbits (A168
and A169) were inoculated with 8.0 log_{10} PFU of vP1015 via
the subcutaneous route on days 0 and 28. Serial bleeds were
taken on days 0, 14, 21, 28, 35, 42 and 49. Sera were
analyzed for the presence of virus neutralizing antibody
using the plaque reduction neutralization method described
by Albrecht et al. (1981).

    Results from this analysis are shown in Table 45. Both
animals seroconverted after a single inoculation of vP1015
with animal A169 exhibiting protective levels of antibody by
two weeks post-inoculation. A significant boost in antibody
levels was observed following a second inoculation. The
results of inoculation of two rabbits with NYVAC-MV (vP913;
Example 9) in an equivalent protocol are included in Table
45. The results indicate that deletion of K2L and K3L did
not affect the ability of the virus to function as an
effective immunization vehicle.

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Table 44. Efficacy of vP1006 in mice.

Virus	187 XP12		vP879		vP1006		vP954	
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
	6.7	8/10	6.7	7/10	6.1	6/10	6.25	0/10
	4.7	9/10	4.7	7/10	4.1	6/10	4.25	0/10
	2.7	3/10	2.7	1/10	2.1	0/10	2.25	0/10
	0.7	3/10	0.7	0/10	0.1	0/10	0.25	0/10
PD_{50}	2.60		4.12		5.25		ND	

[1]dose $TCID_{50}$ log 10/ml

[2]surviving mice/total mice challenged

[3]Kieny et al. (1984)

ND- not detected

Table 45. Comparison of levels of measles virus neutralizing antibody in sera of rabbits inoculated with vP1015 and vP913.

				Titer at Days Post-inoculation			
Animal	0[a]	14	21	28[b]	35	42	49
Animals receiving vP1015							
A168	<1.3[d]	<1.3	<1.3	1.3[c]	2.2	2.2	2.2
A169	<1.3	1.6	1.6	1.6	3.1	3.1	2.5
Animals receiving vP913							
A116	<1.3	<1.3	N.D[e]	<1.3	2.8	2.2	2.2
A117	<1.3	<1.3	N.D.	<1.3	1.9	1.9	1.9

[a] Day of inoculation with 8.0 $\log_{10}$ pfu of vP1015 or vP913 by the sc route

[b] Day of re-inoculation

[c] Titer expressed as $\log_{10}$ of reciprocal of highest dilution showing a 50% reduction in plaque number as compared to pre-inoculation serum.

[d] Lowest dilution tested was 1:20

[e] Not done

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Example 69 - **USE OF NYVAC.2 (vP938) AS A VECTOR;**
                 GENERATION OF VACCINIA RECOMBINANT VP996
                 CONTAINING THE RABIES GLYCOPROTEIN G GENE

In NYVAC.2 (vP938) (Example 17) the [C7L-K1L] deletion
in NYVAC (vP866) is expanded in both directions to include a
total of 38 ORFs [C23L - F4L]. Donor plasmid pRW842
(Example 7), which contains an expression cassette for the
rabies glycoprotein inserted in the TK deletion locus, was
used in recombination with vP938 rescuing virus.
Recombinant vaccinia virus vP996 was identified by plaque
hybridization using ^{32}P-labelled DNA probe to rabies
glycoprotein G coding sequences.

Growth of Modified NYVAC Viruses on Vero and MRC-5
Cells. To examine the effect of the large deletions near
the left [C23L-F4L] and right [B13R-B29R] termini of
vaccinia on growth in tissue culture, NYVAC (vP866), vP938,
vP996 and the following additional NYVAC-based viruses were
tested for replication on MRC-5 and Vero cells.

vP879 (Example 7) contains the rabies glycoprotein gene
inserted into the TK locus of NYVAC.

vP953 (Example 17) contains the NYVAC deletions and an
additional deletion [B13R-B29R] near the right terminus of
the genome.

vP977 (Example 17) contains the NYVAC deletions plus
the additional deletions near both the left and right
termini of the genome.

Vero cells (African Green Monkey kidney cell line, ATCC
CCL 81) and MRC-5 cells (Human embryonic lung, ATCC CCL 171)
were seeded 2 days before infection in 60 mm dishes at
1.5×10^6. At the time of infection all cells were confluent,
the dishes containing 2×10^6 cells. The cells were infected
with virus (Table 46) at a multiplicity of 0.1 pfu per cell,
diluted in MEM+2% newborn calf serum (NCS) with or without
ara C (final concentration 40µg/ml). The infected
monolayers were incubated for 60 min at 37°C with occasional
rocking. After the one hour adsorption period, cells were
washed twice with MEM+2% NCS and then overlayed with 3 ml of
MEM+2% NCS with or without ara C, and incubated at 37°C.
The infected cells were harvested at 1 or 72 hours post-
infection by three cycles of freezing and thawing. Each

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sample was titered in duplicate on Vero cells.

As indicated in Table 46, NYVAC (vP866) is capable of limited growth on human MRC-5 cells. Under the conditions tested here (moi of 0.1 pfu/cell, 72 hr incubation) NYVAC showed an increase in titer of approximately two logs. Under these same conditions, modified NYVAC virus containing the deletion near the left (vP938) or right (vP953) terminus of the genome showed an increase in titer of approximately one log. Yield from the modified NYVAC virus (vP977) containing deletions near both termini was decreased relative to input virus, indicating no amplification on MRC-5 cells. In contrast, under these conditions, all viruses tested amplified to approximately the same extent on Vero cells. As indicated in Table 46, the percentage of vaccinia virus yield on MRC-5 cells compared to yield on Vero cells for the various modified NYVAC virus deletion mutants is as follows: vP866 (NYVAC) 3.5%; vP938 (NYVAC.2, left end deletion) 1.42%; vP953 (right end deletion) 0.5%; vP977 (left and right end deletions) 0.06%. Yields of NYVAC-based rabies recombinant vP879 and NYVAC.2-based rabies recombinant vP996 on MRC-5 cells are essentially the same as for their respective parental viruses, indicating that expression of the foreign gene does not affect growth of the recombinant virus in tissue culture.

Table 46

VIRUS	DELETIONS	INSERT	HPI	VERO TITER (pfu/ ml)	MRC TITER (pfu/ ml)	MRC YIELD (% of VERO)
vP866	NYVAC		1	2.9×10^3	4.4×10^3	
			72	3.7×10^6	1.3×10^5	3.5%
			72A	3.8×10^2	1.1×10^3	
vP879	NYVAC	RABIES	1	2.7×10^3	6.1×10^3	
			72	4.3×10^6	1.5×10^5	3.4%
			72A	6.5×10^2	2.1×10^2	
vP938	NYVAC, C23-F4L		1	7.9×10^3	8.8×10^3	
			72	3.8×10^6	5.4×10^4	1.42%
			72A	4.7×10^2	0	
vP996	NYVAC, C23-F4L	RABIES	1	6.6×10^3	7.3×10^3	
			72	4.0×10^6	4.2×10^4	1.05%
			72A	6.2×10^2	0	
vP953	NYVAC, B13-B29		1	4.0×10^3	5.1×10^3	
			72	6.7×10^6	3.1×10^4	0.5%
			72A	5.2×10^2	7.5×10^1	
vP977	NYVAC, C23-F4L, B13-B29		1	2.4×10^3	5.3×10^3	
			72	1.5×10^6	1.0×10^3	0.06%
			72A	1.0×10^2	0	

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Example 70 - cDNA CLONING OF MUMPS F AND HN GENES

The Urabe AM-9 strain of mumps virus is a live,
attenuated virus which is licensed for use as a vaccine in
Europe and Japan. This virus was obtained as a vaccine
preparation (Imovax Oreillons) from Institut Merieux, Marcy
l'Etoile, France. The virus was amplified on VERO cells
(two passages) and total RNA was isolated and purified from
the infected cells (Chirgwin et al., 1979). First strand
cDNA was prepared from this RNA using AMV reverse
transcriptase and random primers (Watson and Jackson, 1985).
Using the published sequence from the RW mumps strain
(Waxham et al., 1987; Waxham et al., 1988), sets of specific
primers were synthesized from the 5' and 3' untranslated
regions of the F and HN genes. These primers were used to
amplify the Urabe AM-9 F and HN genes from the first strand
cDNA using polymerase chain reaction (PCR). The F gene was
amplified using synthetic oligonucleotides RG503 (SEQ ID
NO:366) (5'-TCTGAGCTCGAAAATAGAATTGATCAG-3') and RG494 (SEQ
ID NO:367) (5'-TCTGGTACCTTTCTGAATGCAGGATG-3'). The HN gene
was amplified using RG500 (SEQ ID NO:368) (5'-TCTGAGCTC
CAATACAACACAGAACC-3') and RG501 (SEQ ID NO:369)
(5'-TCTGGTACCGCATTCACTATTACTCA-3'). The 5' primers (RG503
and RG500) were designed with SacI sites and the 3' primers
(RG494 and RG501) with Asp718 sites. The amplified F and HN
fragments were digested with SacI and Asp718 and cloned into
pBluescript SK+ at the SacI and Asp718 sites in the multiple
cloning site. Six F gene clones and five HN gene clones
were DNA sequenced to eliminate any reverse transcriptase or
PCR errors. FIG. 29 provides the consensus F sequence is
represented by pURF3 (SEQ ID NO:370) and FIG. 30 provides
the consensus HN sequence represented by pURHN5 (SEQ ID
NO:371).

**Example 71 - ALVAC DONOR PLASMID CONSTRUCTION:
                MUMPS F AND HN GENES**

The overall design of an ALVAC donor plasmid for the
generation of ALVAC recombinants expressing the mumps F and
HN genes is as follows. The mumps F gene was coupled to the
entomopox 42K promoter (described in Example 59) and the
mumps HN gene was coupled to the vaccinia H6 promoter

(Perkus et al., 1989). The two promoted genes were
configured in a 5' to 5' orientation and inserted into an
ALVAC C3 locus insertion plasmid. Specific details of the
construction are presented in the following paragraphs.

The multiple cloning region of pSPCP3L (described in
Example 32) was changed by adding a 31 bp linker fragment
containing EcoRV, RsrII, SmaI and PstI restriction sites.
The linker was generated by annealing synthetic
oligonucleotides RG560 (SEQ ID NO:372) (5'-TTAGATATCCGGACCG
CCCGGGCTGCAGAAT-3') and RG561 (SEQ ID NO:373) (5'-ATTCTGCAG
CCCGGGCGGTCCGGATATCTAA-3'), then digesting with EcoRV and
PstI. The linker was cloned into the EcoRV and PstI sites
of pSPCP3L to generate pC3LR.

The H6 promoter was amplified by PCR from pRW823 using
synthetic oligonucleotides RG562 (SEQ ID NO:374) (5'-TATG
AATTCCCATGGTTAATTAATTAGTCATC-3') and RG563 (SEQ ID.NO:375)
(5'-TCTCCCGGGCGGATATCGCGATAATG-3'). pRW823 contains the H6
promoter sequences as described by Perkus et al., 1989. The
purified fragment was digested with EcoRI and SmaI and
ligated into the EcoRI and SmaI sites in pC3LR, resulting in
pC3LRVQH6.

The mumps F gene was excised from pURF3 as a
HindIII/Asp718 fragment (F gene ORF minus about 50 codons
from the N-terminus). The ends of this fragment were
repaired using Klenow polymerase, and ligated into pC3LRVQH6
which had been digested with EcoRI and repaired with Klenow
polymerase. Screening for correct orientation yielded
pC3LRFVQH6.

A fragment containing the 42K promoter sequences and
the N-terminal portion of the F gene were amplified by PCR
from pURF3 using synthetic oligonucleotides RG564 (SEQ ID
NO:376) (5'-TAACCATGGTTTATTGGGAAGAATATGATAATATTTTGGGA
TTTCAAAATTGAAAATATATAATTACAATATAAAATGAAGGCTTTTTTTAGTTAC-3')
and RG565 (SEQ ID NO:377) (5'-CCACTGCAGGCGTCATAC-3'). The
purified fragment was digested with NcoI and PstI and
ligated into pC3LRFVQH6 cut with NcoI and PstI. The
resulting plasmid, pC3LRF42KVQH6, was confirmed by DNA
sequence analysis of the inserted fragment.

The mumps HN gene was excised from pURHN5 as a

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SspI/Asp718 fragment (HN gene ORF minus about 35 codons from
the N-terminus). The ends of this fragment were repaired
using Klenow polmerase, and ligated into pC3LRF42KVQH6 cut
with SmaI. Screening for the correct orientation yielded
pFR2A-30.

     A fragment containing the 3' portion of the H6 promoter
and the N-terminal sequence of the HN gene were amplified by
PCR from pURHN5 using synthetic oligonucleotides RG566 (SEQ
ID NO:378) (5'-TTATCGCGATATCCGTTAAGTTTGTATCGTAATGGAG
CCCTCAAAACTC-3') RG567 (SEQ ID NO:379) (5'-AAACCTAAGG
TCATTAAC-3'). The purified fragment was cut with NruI and
Bsu36I and ligated into pFR2A-30 cut with NruI and Bsu36I.
The resulting ALVAC donor plasmid, pC3URFHN, was confirmed
by DNA sequence analysis of the inserted fragment.

Example 72 - GENERATION OF ALVAC RECOMBINANTS

     The donor plasmid pC3URFHN was used in *in vitro*
recombination experiments in CEF cells with ALVAC (CP$_{pp}$) to
yield vCP171 (Taylor et al., 1992). Recombinant viruses
were identified by *in situ* hybridization procedures (Piccini
et al., 1987) using radiolabelled F and HN specific probes.
Recombinant plaques were purified by three rounds of plaque
purification and amplified for expression analysis. By
expression analysis F and HN are expressed.

**Example 73 - CONSTRUCTION OF INSERTION VECTOR CONTAINING
           JAPANESE ENCEPHALITIS VIRUS (JEV) 15aaC, prM,
           E, NS21, NS2A**

     Construction of pRW838 is as described above (see
Example 15).

     pRW838 was digested at the 3' end of the rabies
glycoprotein gene with EcoRI filled in with the Klenow
fragment of DNA polymerase I digested within the H6 promoter
with EcoRV, and treated with alkaline phosphatase and a 3202
bp fragment containing the 5' 103 bp of the H6 promoter,
plasmid origin of replication and C5 flanking arms isolated.
Plasmid JEVL14VC containing JEV cDNA encoding 15 amino acids
C, prM, E, NS1, NS2A in a vaccinia virus donor plasmid
(Mason et al., 1991) (nucleotides 337-4125, Konishi et al.,
1991) was digested with EcoRV in the H6 promoter and SacI in
JEV sequences (nucleotide 2124) and a 1809 bp fragment
isolated. JEVL14VC was digested with EclXI at the EagI site

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following the T5AT, filled in with the Klenow fragment of
DNA polymerase I and digested with SacI in JEV sequences
(nucleotide 2124) generating a 2011 bp fragment. The 1809
bp EcoRV-SacI, 2011 bp SacI-filled EclXI and 3202 bp EcoRV
filled EcoRI fragments were ligated generating JEVCP1.

**Example 74 -   CONSTRUCTION OF C5 INSERTION VECTOR
                  CONTAINING JEV 15aaC, prM, E**

     Plasmid JEV36 was digested within the H6 promoter with
EcoRV and within JEV sequences with SphI (nucleotide 2380)
and a 2065 bp fragment isolated. Plasmid VQH6C5LSP (defined
in Example 44) was digested within the H6 promoter with
EcoRV and within the polylinker with XbaI and ligated to the
2065 bp fragment plus annealed oligonucleotides SP131 (SEQ
ID NO:382) and SP132 (SEQ ID NO:383) (containing a SphI
sticky end, T nucleotide completing the E coding region,
translation stop, a vaccinia early transcription termination
signal (AT5AT; Yuen and Moss, 1987), a second translation
stop, and XbaI sticky end) generating plasmid JEVCP5 which
encodes 15 amino acids C, prM and E under the control of the
H6 promoter between C5 flanking arms.

Example 75 - CONSTRUCTION OF ALVAC-BASED JEV RECOMBINANTS

     JEVCP1 was transfected into ALVAC infected primary CEF
cells to generate the canarypox recombinant vCP107 encoding
15 amino acids C, prM, E, NS1, NS2A. JEVCP5 was transfected
into ALVAC infected primary CEF cells to generate the
canarypox recombinant vCP140 encoding JEV 15 aa C, prM and
E.

SP131 (SEQ ID NO:382) 5'-   C T tga tttttat tga T   -3'
SP132 (SEQ ID NO:383) 3'-GTACG A ACT AAAAATA ACT AGATC-5'
                  SphI   XbaI

**Example 76 - IMMUNOPRECIPITATION OF JEV PROTEINS IN
                  RECOMBINANT INFECTED CELLS**

     Immunoprecipitation experiments were performed as
described previously (Konishi et al., 1991). The E protein
produced in vCP107 and vCP140 infected cells comigrates with
the E protein produced by JEV-vaccinia recombinants which
have been shown to produce an authentic E protein (Konishi
et al., 1991). vCP107 produces an NS1 protein that
comigrates with the NS1 protein produced by JEV-vaccinia

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recombinants which have been shown to produce an authentic NS1 protein (Konishi et al., 1991).

Example 77 - IMMUNIZATION OF MICE WITH vCP107

Three-week-old Swiss mice were immunized by intraperitoneal injection with 10^7 PFU of vCP107 and 3 weeks later sera were collected from selected mice. One half of the mice were reinoculated with vCP107 and sera were collected 3 weeks later. Sera were assayed for neutralizing (Neut) and hemagglutination inhibiting antibodies (HAI) as previously described (Konishi et al., 1991). Mice immunized once or twice with vCP107 developed high titers of Neut and HAI antibodies, both titers were increased by the second immunization. Mice receiving parental ALVAC did not demonstrate antibody titers. The levels of antibodies obtained in vCP107 immunized mice were comparable to those achieved by immunization with JEV-vaccinia recombinants (Konishi et al., 1991).

Example 78 - EFFICACY STUDIES ON TROVAC-NDV (vFP96)

A number of studies have been performed to determine the protective efficacy against NDV (Newcastle Disease virus) of TROVAC-NDV (vFP96) (Example 8) in specific pathogen free (SPF) chickens and in commercial broiler chickens.

Study A. Four groups of one day old SPF chickens were inoculated by the intramuscular route in the leg with a range of doses from 0.3 to 6.3 $\log_{10}$ $TCID_{50}$ of TROVAC-NDV (vFP96) or the parental TROVAC virus. Birds were monitored for reaction at the site of inoculation and serum samples were collected at 14 days post-inoculation for analysis of the presence of anti-NDV serum neutralizing antibody and hemagglutinin-inhibiting antibody. At 21 days post-inoculation, birds were challenged by intramuscular inoculation of 5.0 $\log_{10}$ 50% egg infectious doses of the velogenic NDV strain Texas GB. Birds were maintained for 14 days post-challenge at which time healthy birds were considered protected.

It was determined that intramuscular inoculation of 6.3 or 2.6 $\log_{10}$ $TCID_{50}$ of TROVAC-NDV (vFP96) or TROVAC parental virus led to a cutaneous lesion at the point of inoculation

which in some cases led to death. There was a specific dose
effect on the severity of the lesion, which, at a dose of
1.1 to 1.4 $\log_{10}$ $TCID_{50}$, was limited to slight inflammatory
reaction at the point of inoculation. In all cases the
lesion was limited to the point of inoculation and did not
spread to other parts of the chicken. The effect was not due
to expression of the NDV F and HN genes in TROVAC-NDV as the
same effect was noted with the parental virus. The response
is specific to this route of inoculation as no detrimental
side effects of inoculation of high doses of TROVAC or
TROVAC based recombinants have been seen when other routes
of inoculation are utilized.

The results of serological analysis and protective
efficacy are shown in Table 47. All birds inoculated with
TROVAC parental virus succumbed to the lethal challenge. All
birds vaccinated with 1.1 and 2.6 $\log_{10}$ $TCID_{50}$ of TROVAC-NDV
(vFP96) survived challenge while 82% of birds receiving 0.3
$\log_{10}$ $TCID_{50}$ survived. This indicates that the 50%
protective dose (PD_{50}) for this recombinant by this route is
less than 0.3 $TCID_{50}$.

Table 47. Safety studies and protective efficacy of TROVAC-
NDV in specific pathogen free day old chickens.

Virus	Dose[a]	Antibody[b]		Safety[c]	Protection[d]
		SN	HI	post-vaccination	post-challenge
TROVAC					
	0.3	ND	ND	0/17	17/17
	1.4	ND	ND	0/7	7/7
	2.7	ND	ND	1/17	16/16
	6.2	ND	ND	5/7	2/2
TROVAC-NDV					
	0.3	NT	NT	0/17	3/17
	1.1	NT	NT	0/17	0/7
	2.6	0.8	4.5	0/17	0/17
	6.3	1.1	6.0	3/14	2/11
CONTROLS					
	None	NT	NT		10/10

a: Birds received one inoculation of either TROVAC or TROVAC-NDV by
     the intramuscular route at one day of age. Dose expressed as $\log_{10}$
     $TCID_{50}$.
b: Serum neutralizing (SN) titers and hemagglutination-inhibition
     (HI) titers are expressed as mean of birds tested. SN titer
     expressed as $\log_{10}$ of reciprocal of highest antibody dilution
     showing complete neutralization of cytopathic effect. HI titer
     expressed as $\log_2$ of reciprocal of last antibody dilution showing
     inhibition of agglutination.
c: Ratio of number of birds dying to number of birds vaccinated
d: Ratio of number of birds dying to number of birds challenged.
     Birds were challenged by intramuscular inoculation of 5.0 $\log_{10}$
     EID_{50} of NDV Texas strain GB.
ND: Not detectable
NT: Not tested

Study B. Protective efficacy of TROVAC-NDV (vFP96) in two sources of commercial broiler chickens and in SPF chickens was assessed. Three groups of one day old birds were studied. Group 1: Chickens were hatched from eggs obtained from a hatchery in which there was no prior history of vaccination with fowlpox virus. Group 2: Chickens were hatched from eggs obtained from a hatchery in which there was a prior history of vaccination with fowlpox virus. Group 3: Specific pathogen free chickens were obtained.

Birds were vaccinated subcutaneously with either 2.0 or 4.0 $\log_{10}$ EID$_{50}$ of TROVAC-NDV. Birds of each group were kept as un-vaccinated contact controls. Birds were bled at 21 and 28 days post-vaccination and the presence of NDV HI antibody assessed. Sera were also assessed for the presence of fowlpox antibody post-vaccination using the agar gel precipitin test. At 28 days post-vaccination, 10 birds of each group and 10 controls were challenged by intra-muscular inoculation of 4.2 $\log_{10}$ EID$_{50}$ of the velogenic NDV strain Texas GB and survivors were assessed. Ten birds of each group and 10 controls were also challenged by wing stab inoculation of the NVSL strain of fowlpox virus. Protection from fowlpox challenge was assessed on the basis of lack of a take or lesion at the site of inoculation. Results of this experiment are shown in Table 48.

The results indicate that the commercial broiler chickens did not develop a detectable immune response to NDV antigens expressed by TROVAC-NDV while SPF chickens did show an immune response. Despite the lack of specific anti-NDV antibody in commercial birds, there was no difference in the level of protection seen after a velogenic NDV challenge. None of the birds showed an immune response to fowlpox vaccination detectable by the agar gel precipitin test. Despite this lack of detectable anti-fowlpox antibody all vaccinated birds were protected from fowlpox challenge while non-vaccinated birds succumbed. These results indicate that prior exposure of the hen to fowlpox virus does not prohibit the induction of protective immunity against NDV or fowlpox in chickens vaccinated with a fowlpox based NDV recombinant.

Table 48. Protective efficacy of TROVAC-NDV (vFP96) in SPF and
          commercial broiler chickens.

Bird Group	Dose	NDV HI GMT[d]		Percent Protection[e]	
		Week 3	Week 4	NDV	FP
Group 1[a]	2.0	<5	<5	70	100
	4.0	<5	<5	70	100
	None	<5	<5	0	0
Group 2[b]	2.0	<5	<5	40	100
	4.0	<5	<5	70	100
	None	<5	<5	0	0
Group 3[c]	2.0	17	13	80	100
	4.0	20	11	80	100
	None	<5	<5	0	0

a: Birds from a hatchery with no prior history of vaccination with
    fowlpox virus.

b: Birds from a hatchery with prior history of vaccination with
    fowlpox virus

c: Specific pathogen free birds

d: Geometric mean titer of HI antibody

e: Percent protection of birds after NDV or Fowpox challenge

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Certain Materials and Methods for Following Examples 79
to 81, Which Relate to Human Recombinant Immunodeficiency
Virus-NYVAC or -ALVAC Virus: Cell Lines. P815 murine
mastocytoma cells (H-2^d) were obtained from the American
Type Culture Collection and maintained in Eagle's Minimum
Essential Medium supplemented with 10% fetal bovine serum
(FBS) and 100 IU/ml penicillin and 100 μg/ml streptomycin.

Mice. Female BALB/cJ (H-2^d) mice were purchased from
Jackson Laboratories (Bar Harbor, ME) and maintained on
mouse chow and water *ad libitum*. All mice were used between
the ages of six and fifteen weeks of age.

Inoculations. Mice were intravenously inoculated with
5x10^7 plaque forming units (pfu) in 0.1 ml of phosphate-
buffered saline via the lateral tail vein.

Sera. Mice were lightly anesthetized with ether and
blood was collected from the retroorbital plexus. Blood
from mice comprising an experimental group was pooled and
allowed to clot. The serum was collected and stored at −
70°C until use.

Assay for Cytotoxic T Lymphocytes and *In Vitro*
Stimulation of Memory Precursors of Cytotoxic T Lymphocytes.
Six week old female BALB/c mice were inoculated
intravenously with 5x10^7 pfu of vaccinia virus (NYVAC),
recombinant vaccinia virus expressing HIV-1 (IIIB) env
(vP911), canarypoxvirus (ALVAC), or with recombinant
canarypoxvirus expressing HIV-1 (IIIB) env (vCP112). Seven
days later, the spleen cells were assayed for primary CTL
activity against unmodified P815 cells or P815 cells that
had been incubated overnight with a peptide corresponding to
the hypervariable V3 loop region of HIV-1 (IIIB) gp120.
Twenty-two days after the initial immunization, the spleen
cells of the experimental mice were incubated with poxvirus
infected stimulator spleen cells and assayed for memory CTL
activity against peptide pulsed targets as before. To
determine secondary CTL activity, 29 days after the primary
immunization mice received a second inoculation of identical
dosage and content as the first. Five days later, the
spleen cells were assayed for cytolytic activity against
peptide pulsed targets. For cytotoxicity assays, H-2^d P815

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murine mastocytoma cells were incubated overnight in medium
(Minimum Essential Medium containing Earle's salts and
supplemented with 10% fetal bovine serum, 2 mM L-glutamine,
100 U/ml penicillin, and 100 μg/ml streptomycin) with or
without 20 μg/ml V3 peptide (CNTRKRIRIQRGPGRAFVTGK, American
Bio-Technologies, Inc.) (SEQ ID NO:457). The following
morning, the P815 cells were washed by centrifugation and
labeled for 1 hr at 37°C in 100 μCi of $Na_2^{51}CrO_4$ per $2x10^6$
cells. Intact spleens were aseptically removed from
euthanized mice, bathed in ice cold Hank's Balanced Salt
Solution, and disrupted into single cell suspensions using a
Stomacher blender. The spleen cell suspensions were washed
several times by low speed centrifugation and resuspended in
Assay Medium (RPMI 1640 containing 10% fetal bovine serum,
20 mM HEPES, $5x10^{-5}$ M 2-mercaptoethanol,
100 U/ml penicillin, and 100 μg/ml streptomycin). For
memory CTL activity, the spleen cells from immunized mice
were resuspended in Stimulation Medium (Minimum Essential
Medium with Earle's salts containing 10% fetal bovine serum,
2mM L-glutamine, 10^{-4}M 2-mercaptoethanol, 100 U/ml
penicillin, and 100 μg/ml streptomycin) and stimulated *in
vitro* in upright 25 cm^2 tissue culture flasks with naive
syngeneic stimulator spleen cells that had been infected
with one of the poxviruses or poxvirus recombinants. After
five days at 37°C, the cells were washed, counted, and
resuspended in Assay Medium. ^{51}Chromium labelled target
cells were added to titrated effector cells in 96-well
microtiter plates for a 4 hr ^{51}Cr release assay. Effector
to target cell ratios (E:T) shown for the three assays were
100:1 (primary), 20:1 (memory), and 50:1 (secondary).
Percent cytotoxicity was calculated as (experimental ^{51}Cr
release - spontaneous ^{51}Cr release)/(maximum ^{51}Cr release -
spontaneous ^{51}Cr release) x 100. Maximum release was
determined by the addition of 5% sodium dodecyl sulfate to
target cells while spontaneous release was determined by
incubating target cells in the absence of effector cells.
In none of the experiments presented did spontaneous release
of ^{51}Cr from target cells exceed 20% of maximum ^{51}Cr
release. Error bars represent 1 standard deviation from the

mean. (*) $P<0.05$, Student's t-test compared to appropriate
vaccinia or canarypoxvirus immunized mice.

Cell Surface Phenotype of Cytotoxic Effector Cells.
Methods were essentially mice spleen cells immunized with
vaccinia virus a canarypox virus vectors (NYVAC, ALVAC) or
with vaccinia virus or canarypox virus recombinants
expressing HIV IIIB env (vP911, vCP112). A second
inoculation was administered 30 days after the first. Prior
to addition to V3 peptide pulsed targets, the spleen cells
were treated with monoclonal antibodies or alloantiserum to
murine T-lymphocyte surface antigens in a two-stage
protocol. Briefly, the spleen cells were resuspended at 10^7
viable cells per ml of Cytotoxicity Medium (RPMI 1640
containing 0.2% BSA and 5 mM HEPES) to which was added
alloanti-Thy 1.2 (Cedarlane), monoclonal anti-CD4 (172.4,
the generous gift of K.J. Weinhold, Duke University.Medical
Center), or monoclonal anti-Lyt 2.2 (Cedarlane). After 30
min at 5°C, the cells were washed and resuspended in the
original volume of Cytotoxicity Medium, divided into two
equal portions with or without complement (Rabbit Lo-Tox M,
Cedarlane) and incubated at 37°C for 45 min. The cells were
then washed in Assay Medium and, based on the pre-treatment
cell densities, resuspended in volumes of Assay Medium
approximating effector to target cell ratios of 100:1
(primary), 10:1 (memory), or 80:1 (secondary) before
addition to a 5 hr ^{51}Cr release assay. Error bars represent
1 standard deviation from the means.

Specificity of CTL Antigen Receptor Recognition of the
V3 Loop Region of HIV IIIB gp120. Cytotoxic T lymphocytes
and memory precursors of cytotoxic T lymphocytes were
generated by inoculation of mice with vCP112 as described
above. Assays for cytotoxic T lymphocytes were performed as
described above except that P815 target cells were pulsed
overnight with V3 peptide from HIV-1 IIIB
(CNTRKRIRIQRGPGRAFVTGK) (SEQ ID NO:384), MN
(CNKRKRIHIGPGRAFYTTKN) (SEQ ID NO:385), or SF2
(CNTRKSIYIGPGRAFHTTGR) (SEQ ID NO:386). Effector to target
cell ratios were 100:1 (primary), 20:1 (memory), and 50:1
(secondary).

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Antibody Responses to HIV-1 (IIIB) gp120. The wells of
ELISA plates (Immulon II) were coated overnight at 4°C with
0.5 µg of partially purified HIV-1 (IIIB) gp120 (Dr. G.
Franchini, NCI-NIH) in carbonate buffer, pH 9.6. The plates
were then washed with phosphate-buffered saline containing
0.05% Tween 20 (PBST). The plates were then blocked for 2
hr at 37°C with PBST containing 1% bovine serum albumin
(BSA). After washing with PBST, sera were initially diluted
1:20 with PBST containing 0.1% BSA (dilution buffer). The
sera were further 2-fold serially diluted in the wells of
the ELISA plate. The plates were incubated at 37°C for 2 hr
and washed with PBST. Horseradish peroxidase conjugated
rabbit anti-mouse immunoglobulins (DAKO) was diluted 1:2000
in dilution buffer and added to the wells of the ELISA
plated and incubated at 37°C for 1 hour. After washing with
PBST, OPD (o-phenylenediamine d: /drochloride) in substrate
buffer was added and the color was allowed to develop at
ambient temperature for about 20 min. The reaction was
extinguished by the addition of 2.5 M H_2SO_4. The absorbance
at 490 nm was determined on a Bio-Tek EL-309 ELISA reader.
The serum endpoint was defined as the reciprocal of the
dilution giving an absorbance value of 0.4.

**Example 79 - A RECOMBINANT CANARYPOXVIRUS EXPRESSING
              HIV env ELICITS HIV-SPECIFIC CYTOTOXIC
              T LYMPHOCYTE ACTIVITY**

Seven days after the initial inoculation with the HIV
canarypoxvirus recombinant (vCP112; defined in Example 18),
cytotoxic responses of spleen cells against HIV V3 peptide
pulsed target cells were roughly equivalent to the cytotoxic
responses elicited by the same dose, $5x10^7$ pfu, of a
vaccinia virus recombinant (vP911) (Example 18) expressing
the same HIV env gene (FIG. 31). Following appropriate *in
vitro* stimulation or a second inoculation, the levels of
cytotoxicity of the spleen cells of mice given the
canarypoxvirus recombinant increased and were comparable to
spleen cells from mice similarly administered the vaccinia
virus recombinant. No such cytotoxic responses were
detected from spleen cells of mice inoculated with the non-
recombinant vaccinia virus or canarypoxvirus vectors, NYVAC
and ALVAC, respectively, confirming the requirement for

immunization with a poxvirus recombinant expressing the HIV
env gene. Furthermore, no cytotoxic reactivity was detected
against unmodified P815 cells from the spleen cells of any
of the mice regardless of the inoculation regimen. Thus,
only mice inoculated with recombinant vaccinia virus or,
more significantly, recombinant canarypoxvirus expressing
the env coding sequence from HIV-1 demonstrated V3-specific
cytotoxic responses.

Example 80   -   CHARACTERIZATION OF CYTOTOXIC EFFECTOR CELLS

      To determine the identity of the spleen cells
associated with the lysis of HIV-1 V3 peptide pulsed target
cells, mice were immunized with vCP112. After each
immunization, or in vitro stimulation 21 days after the
first inoculation, a two-step depletion procedure was
performed, and the spleen cells were assessed for
cytotoxicity against V3 peptide pulsed P815 cells. . Mice
inoculated with the canarypox vector ALVAC did not generate
spleen cells capable of killing peptide pulsed targets and
are not shown. Following a single immunization, vCP112
induced spleen cells able to kill V3 peptide pulsed targets.
The lytic effector cells were sensitive to treatment with
anti-murine Thy 1.2 or Lyt 2.2 plus complement and were
resistant to anti-CD4 as shown in FIG. 32 which shows the
sensitivity of the cytotoxic effector cells from spleen
cells of immunized with vCP112 to antibodies against
cytotoxic T lymphocyte cell surface antigens Thy 1.2 and Lyt
2.2. Neither complement nor any of the monoclonal
antibodies or alloantisera alone affected the cytolytic
action of these cells. Similar results were obtained five
days after a second immunization administered on day 30.
Twenty-one days after a single inoculation, in vitro
stimulation with vCP112 infected syngeneic spleen cells gave
rise to lytic effector cells only partially sensitive to
anti-Thy 1.2 although completely sensitive to anti-Lyt 2.2
and resistant to anti-CD4. These Thy 1.2-, CD4-, Lyt 2.2+
effector cells are not seen following in vitro stimulation
with vP911 of spleen cells from vCP112 inoculated mice.
Nonetheless, it is clear that HIV V3 loop specific
cytotoxicity was mediated by a population of T lymphocytes

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expressing Thy 1.2 and Lyt 2.2, but not CD4. This cell
surface phenotype is characteristic of classical cytotoxic T
lymphocytes.

Example 81 - **SPECIFICITY OF CTL ANTIGEN RECEPTOR
                   RECOGNITION OF THE V3 LOOP REGION
                   OF HIV gp120**

     T lymphocyte antigen receptors are exquisitely
sensitive to small alterations in the primary amino acid
sequence of the epitope fragment. The V3 loop region of HIV
gp120 is hypervariable and differs immunologically among HIV
isolates. The hypervariability resides in substitutions and
additions of only a few amino acids. To examine the
specificity of cytotoxic cells generated by the HIV
canarypox virus recombinant, susceptibility to CTL activity
was compared among P815 target cells pulsed with peptides
corresponding the V3 loop region of gp120 of HIV isolates
IIIB , MN, or SF2. HIV specific primary CTL activity was
confined only to P815 target cells pulsed with peptide
corresponding to the V3 loop of HIV isolate IIIB, but not
target cells pulsed with peptides corresponding to the V3
loop region of gp120 of HIV isolates MN or SF2, as shown in
FIG. 33 which illustrates the specificity of cytotoxic T
lymphocyte antigen receptor for the HIV III B hypervariable
V3 loop of gp120, but not for the V3 loop of HIV MN or SF2.
Similar results were obtained with *in vitro* stimulated, HIV
specific memory CTL activity and secondary CTL activity
induced by immunization with the canarypox virus recombinant
vCP112. Thus, HIV specific CTLs elicited by a recombinant
canarypox virus expressing the env gene of HIV isolate IIIB
recognize only target epitopes derived from the same
antigenic isolate. These results clearly indicate the
exquisite specificity of the lymphocyte effector cells
generated by immunization with the HIV canarypox virus
recombinant and eliminate such nonspecific effector
mechanisms as natural killer (NK) cell activity. These
results are in complete agreement with other reports
characterizing the precision of epitope recognition by HIV
V3-specific murine cytotoxic T lymphocytes.

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**Example 82   -   ANTIBODY RESPONSES OF MICE INOCULATED WITH
                    NYVAC- and ALVAC-BASED HIV RECOMBINANTS**

     To evaluate humoral responses to HIV, mice were
immunized at day 0 with a vaccinia virus HIV recombinant or
canarypoxvirus recombinant and received a secondary
immunization at week four. The mice were bled at various
intervals through 20 weeks after the initial immunization.
Pooled sera from each treatment group were assayed for
antibodies to HIV by ELISA employing purified gp120 as
antigen; the results are shown in FIG. 34 which provides the
antibody responses to HIV III B gp120 of mice immunized with
vectors (NYVAC, ALVAC) or with vaccinia virus recombinant
vP911 or canarypox recombinant (vCP112) expressing HIV-1
env, wherein the inverted triangle indicates the time of
administration of the second inoculation. Primary antibody
responses were generally modest, but detectable. Following
the secondary immunization, the antibody titers of mice
immunized with both vP911 and vCP112 increased and peaked at
week six with titers of over 10,000. These antibody titers
remained at approximately the same levels throughout the
duration of the study. Thus, a canarypoxvirus HIV
recombinant, vCP112, was capable of inducing a significant
antibody response.
     Inoculation of mice with a canarypoxvirus expressing
the env gene of HIV-1 elicits spleen cell reactivity with
characteristics of cytotoxic T lymphocytes: the requirement
for immunization, cell surface phenotype, memory, and
elegant epitope specificity. Furthermore, antibody
responses to HIV-1 gp120 are induced by inoculation with
this recombinant canarypoxvirus.

**Example 83   -   DERIVATION OF NYVAC- AND ALVAC-BASED HIV-1
                    RECOMBINANTS EXPRESSION OF HIV-1(MN) env BY
                    ALVAC AND NYVAC**

     HIV-1(MN) env sequences were derived from plasmid
pMN1.8-9 and pMN1.8-10 which contain a 1774 bp and 1803 bp
subfragment from a genomic cDNA clone of HIV-1(MN),
respectively. These plasmids were provided by the
laboratory of Dr. R.C. Gallo (NCI-NIH). A 1,026 bp
KpnI/EcoRI fragment was derived by amplifying these
sequences from pMN1.8-9 by PCR using oligonucleotides HIVMN6

(SEQ ID NO:387) (5'-GGGTTATTAATGATCTGTAG-3') and HIV3B2 (SEQ
ID NO:151) followed by digestion with KpnI/EcoRI. This
fragment was inserted into pBS-SK digested with KpnI and
EcoRI to yield pBSMIDMN.

   A 1,028 bp SalI/XbaI fragment was derived from pMN1.8-
10 by PCR using oligonucleotides HIVMN5 (SEQ ID NO:388)
(5'-ATCATCGAGCTCTGTTCCTTGGGTTCTTAG-3') and HIVMN3P (SEQ ID
NO:389) (5'-ATCATCTCTAGAATAAAAATTATAGCAAAGCCCTTTCCAAGCC-3')
followed by digestion with SacI and XbaI. This fragment was
co-ligated into pBS-SK digested with EcoRI and XbaI with a
404 bp EcoRI/SacI fragment. The 404 bp fragment was derived
by PCR with pMN1.8-9 as template and oligonucleotides HIV3B1
(SEQ ID NO:144) and HIVMN4 (SEQ ID NO:390) (5'-ATCATCGAGCTCC
TATCGCTGCTC-3'). The resultant plasmid was designated as
pBS3MN.

   The 1.026 bp EcoRI/KpnI fragment from pBSMIDMN was
inserted into the 4,315 bp pBS3MN digested with EcoRI/KpnI
to generate pBSMID3MN. This plasmid contains most of the
env gene except the 5'-most region. The vaccinia virus H6
promoter (Goebel et al., 1990a,b) and the 5'-most region of
the env gene were obtained by isolating a 318 bp KpnI
fragment from pH6HIIIBE (defined in Example 18). This
fragment was ligated into KpnI/XbaI digested pBS-SK along
with the 2.9 bp KpnI/XbaI fragment from pBSMID3MN. The
resultant plasmid was designated as pH6HMNE.

   The 2.7 kb NruI/XbaI fragment from pH6HMNE, containing
the entire HIV-1(MN) env gene juxtaposed 3' to the 3'-most
26 bp of the H6 promoter, was blunt-ended and inserted into
NruI/SmaI digested pSPHAH6. This generated plasmid
pHAHIVMNE. Plasmid pSPHAH6 was derived as follows.

   Plasmid pMP2VCL (containing a polylinker region within
vaccinia sequences upstream of the K1L host range gene) was
digested within the polylinker with HindIII and XhoI and
ligated to annealed oligonucleotides SPHPRHA A through D

(SPHPRHA A (SEQ ID NO:391) 5'-AGCTTCTTTATTC
TATACTTAAAAAGTGAAAATAAATACAAAGGTTCTTGAGGGT-3'
SPHPRHA B (SEQ ID NO:392) 5'-TGTGTTAAATTGAAAGCGAGAAATAATCAT
AAATTATTTCATTATCGCGATATCCGTTAAGTTTGTATCGTAC-3'
SPHPRHA C (SEQ ID NO:393) 3'-TTATTAGTATTTAATAAAGTAATAGCG
CTATAGGCAATTCAAACATAGCATGAGCT-5'
SPHPRHA D (SEQ ID NO:394) 3'-AGAAATAAGATATGAATTTTTCACTTT
TATTTATGTTTCCAAGAACTCCCAACACAATTTAACTTTCGCTCT-5') generating
pSP126 containing a HindIII site, H6 promoter -124 through
-1 (Perkus et al., 1989) and XhoI, KpnI, SmaI, SacI and
EcoRI sites.

     Plasmid pSD544 (containing vaccinia sequences
surrounding the site of the HA gene replaced with a
polylinker region and translation termination codons in six
reading frames) was digested with XhoI within the
polylinker, filled in with the Klenow fragment of DNA
polymerase I and treated with alkaline phosphatase. SP126
was digested with HindIII, treated with Klenow and the H6
promoter isolated by digestion with SmaI. Ligation of the
H6 promoter fragment to pSD544 generated pSPHAH6 which
contained the H6 promoter in the polylinker region (in the
direction of HA transcription). This insertion plasmid
enables the replacement of the vaccinia HA gene (A56; Goebel
et al., 1990a,b) with foreign genetic material.

     The 2.8 kb XbaI/partial KpnI fragment from pH6HMNE was
isolated and inserted into pC5L (defined in Example 44)
digested with XbaI and KpnI. The resultant plasmid was
designated as pC5HIVMNE.

     Plasmids pHAHIVMNE and pC5HIVMNE were used in vitro
recombination experiments with NYVAC (vP866) and
ALVAC(CPpp), respectively, as the rescue virus. These were
done by standard procedures (Piccini et al., 1987). Plaques
derived from recombinant virus were identified by plaque
hybridization using a radiolabeled env-specific DNA probe
(Piccini et al., 1987). After three rounds of plaque
purification, the recombinant viruses were amplified. The
NYVAC-based HIV-1(MN) env recombinant was designated vP1008
and the ALVAC-based recombinant vCP125.

     Recombinant viruses, vCP125 and vP1008, were analyzed

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for expression of the HIV-1(MN) env gene by
immunofluorescence and immunoprecipitation using previously
reported procedures (Taylor et al., 1990). Pooled human
sera from HIV-seropositive individuals (obtained from Dr. K.
Steimer, Chiron Corp., Emeryville, CA) was used in these
assays. Results from immunofluorescence revealed that cells
infected with either vCP125 or vP1008 express the HIV-1(MN)
gene product on their surface. Immunoprecipitation from
lysates prepared from vP1008 and vCP125 infected cells
demonstrated the presence of three predominant HIV-1-
specific proteins with apparent molecular masses of 160 kDa,
120 kDa, and 41 kDa, respectively. These are consistent
with expression of the precursor envelope glycoprotein (160
kDa) and the proteolytically derived mature forms (120 kDa
and 41 kDa).

Example 84 - **EXPRESSION OF THE HIV-1(MN) gp120**
                  BY NYVAC AND ALVAC

A 391 bp EcoRI/XbaI fragment was amplified from pBS3MN
using oligonucleotides T7 (SEQ ID NO:395) (5'-AATACGACTC
ACTATAG-3') and HIVMN120 (SEQ ID NO:396) (5'-ATCATCTCT
AGAATAAAAATTATCTTTTTTCTCTCTGCACCACTC-3') followed by
digestion with EcoRI and XbaI. This fragment was ligated to
the 4.2 kb EcoRI/XbaI fragment derived from pH6HMNE (defined
in Example 83). The resultant plasmid contains a poxvirus
expression cassette for HIV-1(MN) gp120 in pBS-SK and was
designated pBSHIVMN120.

A 1.7 kb XbaI/partial KpnI fragment was isolated and
inserted into pC5L digested with KpnI/XbaI. The resultant
plasmid was designated as pC5HIVMN120. The insertion
plasmid for integrating the HIV-1(MN) gp120 gene into NYVAC
was obtained by first isolating the 1.6 kb NruI/SmaI
fragment from pBSHIVMN120. This fragment was inserted into
pSPHAH6 digested with NruI and SmaI to provide pHAHIVMN120.

Insertion plasmids, pC5HIVMN120 and pHAHIVMN120, were
used in recombination experiments with ALVAC(CPpp) and NYVAC
(vP866) as the rescuing virus. These assays and plaque
identification and purification were performed by standard
procedures (Piccini et al., 1987). Hybridization analyses
were performed with a radiolabeled HIV-1(MN) gp120-specific

probe. Purified recombinants were amplified. The ALVAC-
based HIV-1(MN) gp120 recombinant was designated as vCP124
and the NYVAC-based HIV-1(MN) gp120 recombinant as vP1004.

Cells infected with vCP124 and vP1004 were analyzed for
the presence of the recombinant expressed HIV-1(MN) gp120 by
immunofluorescence and immunoprecipitation. These assays
were performed as previously described (Taylor et al., 1990)
using a pooled human sera from HIV-seropositive individuals
(obtained from K. Steimer, Chiron Corporation, Emeryville,
CA). Results from these studies clearly indicated that
cells infected with either vCP124 and vP1004 contained HIV-
1(MN) gp120, whereas gp120 was not observed in uninfected
cells and cells infected with parental viruses, ALVAC and
NYVAC.

Example 85   -   **EXPRESSION OF A NON-CLEAVABLE FORM OF
HIV-1 gp160 BY ALVAC AND NYVAC**

In order to express a non-cleavable form of the HIV-1
(IIIB) gp160 an arginine to threonine mutation was
engineered at amino acid 511 (Ratner et al., 1985) as was
demonstrated by Guo et al. (1990). These modifications were
made to decrease the shedding of gp120 from the surface of
infected cells. These manipulations were performed as
follows. A 376 bp PstI/XbaI fragment was obtained by first
amplifying the sequences from pH6HIIIBE using
oligonucleotides HIV3B2A (SEQ ID NO:397) (5'-GAAATAA
TAAAACAATAATC-3') and HIVECB (SEQ ID NO:398) (5'-GCT
CCTATTCCCACTGCAGTTTTTTCTCTCTGCAC-3') followed by digestion
with PstI and XbaI. This fragment was ligated with a 1,061
bp PstI/XbaI fragment and a 4.5 kb EcoRI/XbaI fragment from
pH6HIIIBE to yield pBSHIV3BEEC.

The 2.6 kb NruI/XbaI fragment from pBSHIV3BEEC,
containing the 3'-most 26 bp of the H6 promoter linked to
the gp160 cassette, was isolated and ligated to a 3.0 kb
NruI/XbaI fragment of pBSHVS (defined in Example 60) to
yield pBSHIV3BEECM. Digestion with NruI and XbaI excises
the 3'-most 26 bp of the H6 promoter and the Hantaan virus S
sequence. The 3.0 kb NruI/XbaI fragment contains the 5'-
most 100 bp of the H6 promoter in a pBS-SK plasmid.

The 2.8 kb XbaI/partial KpnI fragment from pBSHIV3BEECM

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was ligated to XbaI/KpnI digested pC5L to yield pC5HIV3BEEC.
A 2.7 kb NruI/XbaI fragment from pBSHIV3BEECM was blunt-
ended with the Klenow fragment of the *E. coli* DNA polymerase
and inserted into NruI/SmaI digested pSPHAH6 to yield
pHAHIV3BEEC.

The insertion plasmids, pC5HIV3BEEC and pHAHIV3BEEC,
were used in *in vitro* recombination experiments by standard
procedures (Piccini et al., 1987) using ALVAC(CPpp) and
NYVAC (vP866), respectively, as rescue virus. Recombinant
plaques were identified by standard plaque hybridization
analysis (Piccini et al., 1987) using a radiolabeled probe
specific for the HIV-1 env gene. Recombinant viruses were
amplified following three rounds of purification. The
ALVAC-based HIV-1(IIIB) gp160 (non-cleavable) was designated
as vCP126 and the NYVAC-based equivalent as vP1020.

Immunofluorescence and immunoprecipitation analyses
were performed by previously described procedures (Taylor et
al., 1990) on vP1020 and vCP126 infected cells using pooled
human serum from HIV-seropositive individuals (obtained from
K. Steimer, Chiron Corp., Emeryville, CA).
Immunofluorescence results clearly demonstrated the surface
expression of the HIV-1(IIIB) gp160 (non-cleavable form) on
the surface of cells infected with either vCP126 or vP1020.
Furthermore, immunoprecipitation results demonstrated the
presence of a HIV-1(IIIB) gp160 in these infected cells that
was not proteolytically cleaved into the mature gp120 and
gp41 frames.

A non-cleavable form of the HIV-1(MN) gp160 was also
pursued and the recombinant viruses obtained as follows.

A PstI/XbaI fragment was obtained by PCR amplification
from pH6HMNE using oligonucleotides HIVMN3P (SEQ ID NO:389)
(5'-ATCATCTCTAGAATAAAAATTATAGGAAAGCCCTTTCCAAGCC-3') and
HIVECA (SEQ ID NO:399) (5'-GTGCAGAGAAAAAACTGCAGTGGGAAT
AGGAGC-3') followed by digestion with PstI and XbaI. This
1061 bp fragment was ligated with the 391 bp EcoRI/PstI from
pBSHIVMNT (below) and the 4.2 kb EcoRI/XbaI fragment from
pH6HMNE (defined in Example83). The resultant plasmid was
designated as pBSHIVMNEEC1. Sequence analysis of the HIV
env insert demonstrated that a single nucleotide was

missing. To correct this, the following manipulations were
performed. The 4.6 kb SacI/XbaI from pH6HMNE resulting in
the formation of pBSHIVMNEEC.

The 2.6 NruI/XbaI fragment from pBSHIVMNEEC was
isolated, blunt-ended with Klenow, and inserted into
NruI/SmaI digested pSPHAH6 (defined in Example 83). The
resultant plasmid was designated pHAHIVMNEEC. The 2.6 kb
NruI/XbaI fragment from pBSHIVMNEEC was also inserted into
NruI/XbaI digested pVQH6C5LSP6 (below) to yield pC5HIVMNEEC.

Insertion plasmids, pHAHIVMNEEC and pC5HIVMNEEC, were
used in standard recombination experiments (Piccini et al.,
1987) with NYVAC (vP866) and ALVAC(CPpp), respectively, as
rescue virus. Recombinant virus was identified and plaque
purified by standard plaque hybridization (Piccini et al.,
1987) using a radiolabeled HIV env-specific DNA probe.
Purified rcombinant virus were then amplified. The NYVAC-
based recombinant containing the HIV-1 (MN) non-cleavable
gp160 was designated as vP1078 and the ALVAC equivalent
vCP144.

Expression analysis of vCP126 and vP1078 was performed
as described above. These results demonstrated that
expression was qualitatively equivalent to the HIV-1 (IIIB)
counterparts, vP1020 and vCP126.

**Example 86 - EXPRESSION OF A NON-CLEAVABLE, SECRETED FORM
              OF HIV-1 env BY ALVAC AND NYVAC**

ALVAC- and NYVAC-based recombinant viruses were
generated which express an HIV-1(MN) env that is not
proteolytically cleaved and is secreted by virtue of the
elimination of the transmembrane sequence near the carboxy
terminus of the gene product. A 502 bp PstI/XbaI fragment
was obtained by first amplifying these sequences from
pH6HMNE (defined in Example 83) using oligonucleotides
HIVECA (SEQ ID NO:399) and HIVMNT1 (SEQ ID NO:400) (5'-
ATCATCTCTAGAATAAAAATTACAAACTTGCCCATTTATCCAATTCC-3') followed
by digestion with PstI (5'-end) and XbaI (3'-end). This
fragment corresponds to nucleotides 7219 to 7808 (Ratner et
al., 1985). This fragment will serve as the 3'-end of the
env expression cassette. As such, the env gene product will
lack the transmembrane region, will be terminated by a

termination codon provided by oligonucleotide HIVMNT1 (SEQ
ID NO:400), and will not be cleaved due to an amino change
at 511 (above) provided using oligonucleotide HIVECA (SEQ ID
NO:399). This 502 bp fragment was ligated to the 391 bp
EcoRI/PstI fragment derived by PCR from pH6HMNE using
oligonucleotides HIV3B1 (SEQ ID NO:144) and HIVECB (SEQ ID
NO:398), and the 4.2 kb EcoRI/XbaI fragment to pH6MNE. The
resultant plasmid was designated pBSHIVMNT.

The 2.2 kb XbaI/partial KpnI fragment from pBSHIVMNT
was isolated and inserted into pC5L digested with XbaI and
KpnI. The resultant plasmid was designated as pC5HIVMNT.
The NYVAC insertion plasmid was derived by isolating the 2.1
kb NruI/XbaI fragment from pBSHIVMNT. This fragment was
then blunt-ended with the Klenow fragment of the E. coli DNA
polymerase in the presence of 2mM dNTPs and inserted into
pSPHAH6 digested with NruI and SmaI to yield pHAHIVMNT.

The insertion plasmids, pC5HIVMNT and pHAHIVMNT, were
used in standard recombination experiments (Piccini et al.,
1987) with ALVAC(CPpp) and NYVAC (vP866), respectively, as
the rescue virus. Recombinant virus was identified by
standard plaque hybridization assays (Piccini et al., 1987)
using a radiolabeled HIV env-specific probe. Recombinant
virus was subjected to three rounds of purification prior to
amplification. The ALVAC-based HIV-1(MN) env (non-
cleavable; secreted) was designated as vCP120 and the NYVAC
equivalent as vP994.

Immunoprecipitation analyses were performed as
previously described (Taylor et al., 1990) for vCP120 and
vP994 infected cells using pooled human sera from HIV-
seropositive individuals. Both vCP120 and vP994 expressed
an HIV-1(MN) env-specific gene product with a molecular
weight consistent with a non-cleavable, truncated gene
product. Furthermore, immunoprecipitation of the cell-free
medium from vCP120 and vP994 infected cell cultures
indicated the secretion of this env gene product.

A similar construction was engineered for the HIV-
1(IIIB) env. The following manipulations were performed to
accomplish this. A 487 bp PstI/XbaI fragment was obtained
by first amplifying these sequences from pH6HIIIBE (defined

in Example 18) using oligonucleotides HIVECA (SEQ ID NO:399)
and HIV3BT (SEQ ID NO:401) (5'-ATCATCTCTAGAATAAAAATTACAAACT
TGCCCATTTATCTAATTCC-3') followed by digestion with PstI and
XbaI. A 397 bp EcoRI/PstI fragment was isolated from
pBSHIV3BEEC and a 4.2 kb EcoRI/XbaI fragment was isolated
from pH6HIIIBEM. These three fragments were ligated
together to yield pBSHIV3BT1.

The 2.1 kb and 2.9 kb fragments derived by HindIII/XbaI
digestion of pBSHIV3BEECM were ligated to the 105 bp
HindIII/XbaI fragment from pBSHIV3BT1 to yield pBSHIV3BT.
This plasmid was digested with NruI and XbaI to excise a 2.1
kb fragment. This fragment was blunt-ended and inserted
into pSPHAH6 digested with NruI and SmaI to generate
pHAHIV3BT.

The plasmid pHAHIV3BT was used in recombination
experiments, as above, with NYVAC (vP866) as the rescue
virus. Recombinant virus was identified and purified as
above and the resultant recombinant was designated as
vP1036. This recombinant had all the expression
characteristics noted above for vCP120 and vP994.

**Example 87 - EXPRESSION OF HIV-1(MN) gp120 ANCHORED WITH
A TRANSMEMBRANE SEQUENCE BY NYVAC AND ALVAC**

To fuse the env region encoding the gp120 to the region
encoding the hydrophobic transmembrane sequence, the
following manipulations were performed. A 200 bp fragment
corresponding to the 3'-most region of the gp120 coding
sequence was derived by PCR from pH6HMNE (defined in
Example 83) using oligonucleotides HIV3B1 (SEQ ID NO:144)
and HIVMN18 (SEQ ID NO:402) (5'-GCCTCCTACTATCATTATGAATAAT
CTTTTTCTCTCTG-3'). This fragment was fused by PCR to
annealed oligonucleotides HIVTM1 (SEQ ID NO:403) (5'-
TTATTCATAATGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGCTGTACTCTCT
GTAGTGAATAGAGTTAGGCAGGGATAA-3') and HIVTM2 (SEQ ID NO:404)
(5'-TTATCCCTGCCTAACTCTATTCACTACAGAGGTACAGCAAAAACTATTCTTAAAC
CTACCAAGCCTCCTACTATCATTATGAATAA-3') using oligonucleotides
HIV3B1 (SEQ ID NO:144) and HIVTM3 (SEQ ID NO:405) (5'-ATCA
TCTCTAGAATAAAAATTATCCCTGCCTAACTCTATTCAC-3').
Oligonucleotides HIVTM1 (SEQ ID NO:403) and HIVTM2 (SEQ ID
NO:404) correspond to nucleotides 7850 to 7934 (Ratner et

al., 1985) and represent the region encoding the HIV env
hydrophobic anchor sequence. Fusion with HIVTM3 (SEQ ID
NO:405) engineers the 3'-end of the eventual cassette with a
termination codon and a 3' XbaI site. The derived fragment
was digested with EcoRI/XbaI and ligated to pH6HMNE digested
with EcoRI and XbaI to yield pBSHIVMN120T.

     The 1.7 bk NruI/XbaI fragment from pBSHIVMN120T,
containing the 3'-most 26 bp of the H6 promoter and the
entire HIV-1 cassette, was isolated and inserted into the
5.1 kb NruI/XbaI fragment from pVQH6C5LSP6 to derive
pC5HIVMN120T. The plasmid pVQH6C5LSP6 was derived as
follows.

     pC5LSP (defined in Example 66) was digested with BamHI
and ligated to annealed oligonucleotides CP32 (SEQ ID
NO:406) (5'-GATCTTAATTAATTAGTCATCAGGCAGGGCGAGAACGA
GACTATCTGCTCGTTAATTAATTAGGTCGACG-3') and CP33 (SEQ ID
NO:407) (5'-GATCCGTCGACCTAATTAATTAACGAGCACATAGTCTCGTTCTCGCC
CTGCCTGATGACTAATTAATTAA-3') to generate pVQC5LSP6.

     The 1.7 kb NruI/XbaI fragment from pBSHIVMN120T was
also blunt-ended and inserted into pSPHAH6 digested with
NruI and SmaI. The resultant plasmid was designated as
pHAHIVMN120T.

     Insertion plasmids, pC5HIVMN120T and pHAHIVMN120T, were
used in standard recombination experiments (Piccini et al.,
1987) with ALVAC and NYVAC, respectively, as the rescue
virus. Recombinant virus was identified and purified by
standard plaque hybridization (Piccini et al., 1987) using a
radiolabeled HIV-1 gp120-specific DNA probe. The pure
populations were amplified and the ALVAC-based anchored HIV-
1(MN) gp120 recombinant was designated vCP138. The NYVAC-
based equivalent was designated vP1035.

     Immunofluorescence and immunoprecipitation analyses
were performed by standard procedures (Taylor et al., 1990)
to evaluate expression of the HIV-1(MN) anchored gp120 in
vP138 and vP1035 infected cells. The assays were performed
using pooled human sera from HIV-seropositive individuals
(obtained from Dr. K. Steimer, Chiron Corp., Emeryville,
CA). Investigation of surface immunofluorescence indicated
that vCP138 and vP1035 infected cells contained HIV-1(MN)

gp120 in the plasma membrane. Significantly, the surface
staining of vCP138 and vP1035 infected cells was greatly
enhanced compared to cells infected with recombinant viruses
(i.e. vCP125, vCP124, vP1004, and vP1008) expressing gp160
or a non-anchored gp120. Results from immunoprecipitation
analyses confirmed the expression of gp120 in vCP138 and
vP1035 infected cells and that the expressed product was of
the expected molecular mass.

Example 88   -   **GENERATION OF NYVAC/HIV-1 GAG (PROTEASE⁻)
                     RECOMBINANT**

     A plasmid, pHXB2D, containing human immunodeficiency
virus type 1 (HIV-1) cDNA sequence was obtained from Dr.
R.C. Gallo (NCI-NIH) (NCI-NIH). The sequence encoding the
5'-end of the gag gene was cloned between vaccinia virus tk
flanking arms. This was accomplished by cloning the 1,625
bp BglII fragment of pHXB2D, containing the 5'-end of the
gag gene, into the 4,075 bp BglII fragment of pSD542
(defined in Example 32). The plasmid generated by this
manipulation is called pHIVG2.

     The 3'-end of the gag gene was then cloned downstream
from the rest of the gag gene. This was accomplished by
cloning a 280 bp ApaI-BamHI PCR fragment, containing the 3'-
end of the gag gene, into the 5,620 bp ApaI-BamHI fragment
of pHIVG2. This PCR fragment was generated from the
plasmid, pHXB2D, with the oligonucleotides, HIVP5 (SEQ ID
NO:408) (5'-TGTGGCAAAGAAGGGC-3') and HIVP6 (SEQ ID NO:409)
(5'- TTGGATCCTTATTGTGACGAGGGGTC-3'). The plasmid generated
by this manipulation is called pHIVG3.

     The I3L promoter was then cloned upstream of the gag
gene. This was accomplished by cloning the
oligonucleotides, HIVL17 (SEQ ID NO:410) (5'- GATCTTGAGA
TAAAGTGAAAATATATCATTATATTACAAAGTACAATTATTTAGGTTTAATCATGGGT
GCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGAT-3') and HIVL18 (SEQ ID
NO:411) (5'- CGATCTAATTCTCCCCCGCTTAATACTGACGCTCTCGCACC
CATGATTAAACCTAAATAATTGTACTTTGTAATATAATGATATATATTTTCACTTTATCT
CAA-3'), encoding the vaccinia virus I3L promoter and the
5'-end of the gag gene, into the 5,540 bp partial BglII-ClaI
fragment of pHIVG3. The plasmid generated by this
manipulation is called pHIVG4.

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pHIVG4 was used in recombination experiments with vP866 (NYVAC) as the rescuing virus to yield vP969.

Immunoprecipitation analysis was performed to determine whether vP969 expresses authentic HIV-1 gag precursor protein. Vero cell monolayers were either mock infected, infected with the parental virus or infected with vP969 at an m.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls of modified Eagle's medium (minus methionine) containing 2% fetal bovine serum and [^{35}S]-methionine (20 μCi/ml). Cells were harvested at 18 hrs post-infection by the addition of 1 ml 3X buffer A (3% NP-40, 30mM Tris (pH7.4), 3mM EDTA, 0.03% Na Azide and 0.6 mg/ml PMSF) with subsequent scraping of the cell monolayers. Lysates from the infected cells were analyzed for HIV-1 gag precursor expression using pooled serum from HIV-1 seropositive individuals (obtained from Chiron, Emeryville, CA). The sera was preadsorbed with vP866 infected Vero cells. The preadsorbed sera was bound to Protein A-sepharose in an overnight incubation at 4°C. Following this incubation period, the material was washed 4X with 1X buffer A. Lysates precleared with normal human sera and protein A-sepharose were then incubated overnight at 4°C with the HIV-1 seropositive human sera bound to protein A-sepharose. After the overnight incubation period, the samples were washed 4X with 1X buffer A and 2X with a LiCl$_2$/urea buffer. Precipitated proteins were dissociated from the immune complexes by the addition of 2X Laemmli's buffer (125 mM Tris (pH6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5 min. Proteins were fractionated on a 10% Dreyfuss gel system (Dreyfuss et al., 1984), fixed and treated with 1M Na-salicylate for fluorography.

Human sera from HIV-1 seropositive individuals specifically precipitated the HIV-1 gag precursor protein from vP969 infected cells, but did not precipitate HIV-1-specific proteins from mock infected or NYVAC infected cells.

Example 89 - GENERATION OF NYVAC/HIV-1 gag/pol RECOMBINANT

    The sequence encoding the 5'-end of the gag gene was
cloned between vaccinia virus tk flanking arms. This was
accomplished by cloning the 1,625 bp BglII fragment of
pHXB2D, containing the 5'-end of the gag gene, into the
4,075 bp BglII fragment of pSD542 (defined in Example 32).
The plasmid generated by this manipulation is called pHIVG2.

    The 3'-end of the gag gene was then cloned into pHIVG2.
This was accomplished by cloning a 280 bp ApaI-BamHI PCR
fragment, containing the 3'-end of the gag gene, into the
5,620 bp ApaI-BamHI fragment of pHIVG2. This PCR fragment
was generated from the plasmid, pHXB2D, with the
oligonucleotides, HIVP5 (SEQ ID NO:408) and HIVP6 (SEQ ID
NO:409). The plasmid generated by this manipulation is
called pHIVG3.

    The I3L promoter was then cloned upstream of the gag
gene. This was accomplished by cloning the
oligonucleotides, HIVL17 (SEQ ID NO:410) and HIVL18 (SEQ ID
NO:411), encoding the vaccinia virus I3L promoter and the
5'-end of the gag gene, into the 5,540 bp partial BglII-ClaI
fragment of pHIVG3. The plasmid generated by this
manipulation is called pHIVG4.

    The portion of the gag gene encoding p24, p2, p7 and p6
was then eliminated. This was accomplished by cloning the
oligonucleotides, HIVL19 (SEQ ID NO:412) (5'-CTGA
CACAGGACACAGCAATCAGGTCAGCCAAAATTACTAATTTTTATCTCGAGGTCGACAGGA
CCCG-3') and HIVL20 (SEQ ID NO:413) (5'-GATCCGGGT
CCTGTCGACCTCGAGATAAAAATTAGTAATTTTGGCTGACCTGATTGCTGTGTCCTGTGT
CAG-3'), into the 4,450 bp partial PvuII-BamHI fragment of
pHIVG4. The plasmid generated by this manipulation is
called pHIVG5.

    The remainder of the gag gene, as well as the pol gene,
was then cloned downstream of the p17 "gene". This was
accomplished by cloning the 4,955 bp ClaI-SalI fragment of
pHXB2D, containing most of the gag gene and all of the pol
gene, into the 4,150 bp ClaI-SalI fragment of pHIVG5. The
plasmid generated by this manipulation is called pHIVG6.

    Extraneous 3'-noncoding sequence was then eliminated.
This was accomplished by cloning a 360 bp AflII-BamHI PCR

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fragment, containing the 3'-end of the <u>pol</u> gene, into the
8,030 bp AflII-<u>BamHI</u> fragment of pHIVG6. This PCR fragment
was generated from the plasmid, pHXB2D, with the
oligonucleotides, HIVP7 (SEQ ID NO:414) (5'-AAG
AAAATTATAGGAC-3') and HIVP8 (SEQ ID NO:415) (5'-TTGG
ATCCCTAATCCTCATCCTGT-3'). The plasmid generated by this
manipulation is called pHIVG7.

     pHIVG7 was used in recombination experiments with vP866
(NYVAC) as the rescuing virus to yield vP989.

     Immunoprecipitation experiments with vP989 infected
cells were performed as described above for the expression
of the HIV-1 <u>gag</u> precursor protein. No HIV-1-specific
species were precipitated from mock infected or NYVAC
infected Vero cells. Protein species corresponding to the
<u>gag</u> precursor protein, as well as various intermediate and
mature <u>gag</u> cleavage products, were precipitated, however,
from lysates of vP989 infected cells.

Example 90 - **GENERATION OF NYVAC/HIV-1 gag/pol AND**
                    env (gp120) RECOMBINANT

     The sequence encoding the 5'-end of the <u>gag</u> gene was
cloned between vaccinia virus tk flanking arms. This was
accomplished by initially preparing plasmid pHIVG7, as
described above (<u>see</u> Example 89).

     pHIVG7 was used in recombination experiments with vP921
as the rescuing virus to yield vP991.

     Immunoprecipitation experiments with vP991 infected
cells were performed as described above for the expression
of the HIV <u>gag</u> precursor protein. No HIV-specific species
were precipitated from mock infected Vero cells. Protein
species corresponding to the env and <u>gag</u> precursor proteins,
as well as various intermediate and mature <u>gag</u> cleavage
products, were precipitated, however, from lysates of vP991
infected cells.

Example 91 - **GENERATION OF NYVAC/HIV-1 gag/pol AND**
                    env (gp160) RECOMBINANT

     The sequence encoding the 5'-end of the <u>gag</u> gene was
cloned between vaccinia virus tk flanking arms. This was
accomplished by initially preparing plasmid pHIVG7, as
described above (<u>see</u> Example 89).

pHIVG7 was used in recombination experiments with vP911 (above) as the rescuing virus to yield vP990.

Immunoprecipitation experiments with vP990 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected Vero cells. Protein species corresponding to the env and gag precursor proteins, as well as various intermediate and mature gag cleavage products, were precipitated, however, from lysates of vP990 infected cells.

**Example 92 - GENERATION OF NYVAC/HIV-1 p17, p24
                 RECOMBINANT**

A plasmid, pHXB2D, containing HIV-1 cDNA sequence, was obtained from Dr. R.C. Gallo (NCI-NIH). The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing pHIVG5, as described above (see Example 89).

The 3'-end of the p24 "gene" was then cloned into pHIVG5. This was accomplished by cloning a 660 bp SalI-BamHI PCR fragment, containing the 3'-end of the p24 "gene", into the 4,450 bp SalI-BamHI fragment of pHIVG5. This PCR fragment was generated from the plasmid, pHXB2D, with the oligonucleotides, HIVP25 (SEQ ID NO:416) (5'-AAAGT CGACCCATATCACCTAGAAC-3') and HIVP26 (SEQ ID NO:417) (5'-TTTGGATCCTTACAAAACTCTTGCCTTAT-3'). The plasmid generated by this manipulation is called pHIVG8.

The entomopox 42 kd promoter was then cloned upstream of the p24 "gene". This was accomplished by cloning the oligonucleotides, HIVL21 (SEQ ID NO:418) (5'-TCGAGCAAAAT TGAAAATATATAATTACAATATAAAATGCCTATAGTGCAGAACATCCAGGGGCAAATGGT ACATCAGGCCATATCACCTAGAACTTTAAATGCA-3') and HIVL22 (SEQ ID NO:419) (5'-TTTAAAGTTCTAGGTGATATGGCCTGATGTACCATTTGCCCCTG GATGTTCTGCACTATAGGCATTTTATATTGTAATTATATATTTTCAATTTTGC-3'), encoding the entomopox 42 kd promoter and the 5'-end of the p24 "gene", into the 5,070 bp XhoI-NsiI fragment of pHIVG8. The plasmid generated by this manipulation is called pHIVG9.

pHIVG9 was used in recombination experiments with vP866 (NYVAC) as the rescuing virus to yield vP970.

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Immunoprecipitation experiments with vP970 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected or NYVAC infected Vero cells. A protein species corresponding to p24 was precipitated, however, from lysates of vP970 infected cells.

Example 93 - GENERATION OF NYVAC/HIV-1 p17, p24 AND env (gp120) RECOMBINANT

A plasmid, pHXB2D, containing HIV-1 cDNA sequence, was obtained from Dr. R.C. Gallo (NCI-NIH). The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVG9 as described above (see Example 92).

pHIVG9 was used in recombination experiments with vP921 as the rescuing virus to yield vP973.

Immunoprecipitation experiments with vP973 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected Vero cells. Protein species corresponding to env and p24 were precipitated, however, from lysates of vP973 infected cells.

Example 94 - GENERATION OF NYVAC/HIV-1 p17, p24 AND env (gp160) RECOMBINANT

A plasmid, pHXB2D, containing HIV-1 cDNA sequence, was obtained from Dr. R.C. Gallo (NCI-NIH). The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVG9, as described above (see Example 92).

pHIVG9 was used in recombination experiments with vP911 as the rescuing virus to yield vP971.

Immunoprecipitation experiments with vP971 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected Vero cells. Protein species corresponding to env and p24 were precipitated, however, from lysates of vP971 infected cells.

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**Example 95 - GENERATION OF NYVAC/HIV-1 gag (PROTEASE⁻) AND
                env (TRUNCATED) RECOMBINANT**

A plasmid, pHXB2D, containing HIV-1 cDNA sequence was
obtained from Dr. R.C. Gallo (NCI-NIH). The sequence
encoding the 5'-end of the gag gene was cloned between
vaccinia virus tk flanking arms. This was accomplished by
initially preparing plasmid pHIVG-9 as described above (see
Example 89).

An H6-promoted truncated HIV-1 envelope gene was then
inserted into pHIVG4. This was accomplished by cloning the
E. coli DNA polymerase I (Klenow fragment) filled-in 1,600
bp XhoI-NotI fragment of pHIVE10, containing an H6-promoted
truncated HIV-1 envelope gene, into the filled-in BamHI site
of pHIVG4. The plasmid generated by this manipulation is
called pHIVGE11.

The plasmid pHIVE10 was derived by inserting a
SacI/partial KpnI fragment from pBSHIV3BCDT1 into the
multiple cloning region of pIBI25 (IBI, New Haven, CT). The
plasmid pBSHIV3BCDT1 contains an H6 promoted cassette to
express a severely truncated form of the HIV-1(IIIB)
envelope (amino acid 1 to 447; Ratner et al., 1985).
Expression of this cassette was evaluated to eliminate CD4
binding while retaining the V3 loop region and the T1
epitope.

To construct pBSHIV3BCDT1 the following manipulations
were performed. A PCR-derived fragment of 200 bp was
amplified from pH6HIIIBE (defined in Example 18) using
oligonucleotides HIV3B2A (SEQ ID NO:397) and HIVCD4A (SEQ ID
NO:420) (5'-GCCTCCTACTATCATTATGAATAAACTGATGGGAGGGGCATAC-3').
This fragment was fused by PCR to annealed oligonucleotides
HIVTM1 (SEQ ID NO:403) and HIVTM2 (SEQ ID NO:404) using
oligonucleotides HIV3B2A (SEQ ID NO:397) and HIVTM3 (SEQ ID
NO:405). These manipulations create the 3'-end of the
truncated env cassette by placing sequences encoding the
HIV-1 env transmembrane anchor (amino acids 691 to 718;
Ratner et al., 1985), a translation termination codon (TAA),
and a 3' XbaI site. This PCR-fusion product was digested
with EcoRI and XbaI to yield a 243 bp fragment. The
fragment was ligated to the 4.5 bp EcoRI/XbaI fragment of

pH6HIIIBE to generate pBSHIV3BCDT1.

pHIVGE11 was used in recombination experiments with vP866 (NYVAC) as the rescuing virus to yield vP979.

Immunoprecipitation experiments with vP979 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected or NYVAC infected Vero cells. Protein species corresponding to env and the gag precursor proteins were precipitated, however, from lysates of vP979 infected cells.

Example 96 - **GENERATION OF NYVAC/HIV-1 gag/pol AND env (TRUNCATED) RECOMBINANT**

The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVG7 as described above (see Example 89).

An H6-promoted truncated HIV-1 envelope gene was then inserted into pHIVG7. This was accomplished by cloning the *E. coli* DNA polymerase I (Klenow fragment) filled-in 1,600 bp XhoI-NotI fragment of pHIVE10 (defined in Example 95), containing an H6-promoted truncated HIV-1 envelope gene, into the filled-in BamHI site of pHIVG7. The plasmid generated by this manipulation is called pHIVGE12.

pHIVGE12 was used in recombination experiments with vP866 (NYVAC) as the rescuing virus to yield vP978.

Immunoprecipitation experiments with vP978 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected or NYVAC infected Vero cells. Protein species corresponding to the env and gag precursor proteins, as well as various intermediate and mature gag cleavage products, were precipitated, however, from lysates of vP978 infected cells.

Example 97 - **GENERATION OF NYVAC/HIV-1 gag/pol AND env (gp120) RECOMBINANT**

The sequence encoding the the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVG7, as described above (see Example 89).

The I3L-promoted gag and pol genes were then inserted
into a canary pox insertion vector. This was accomplished
by cloning the 4,360 bp partial BglII-BamHI fragment of
pHIVG7, containing the I3L-promoted gag and pol genes, into
the BamHI site of pVQH6CP3L. The plasmid generated by this
manipulation is called pHIVGE14.

The H6-promoted HIV-1(MN) envelope (gp120) gene was
then inserted into pHIVGE14. This was accomplished by
cloning the oligonucleotides, HIVL29 (SEQ ID NO:421)
(5'-GGCCGCAAC-3') and HIVL30 (SEQ ID NO:422)
(5'-TCGAGTTGC-3'), and the 1,600 bp NruI-NotI fragment of
pBSHIVMN120, containing the H6-promoted gp120 gene, into the
11,500 bp NruI-XhoI fragment of pHIVGE14. The plasmid
generated by this manipulation is called pHIVGE15.

The H6-promoted envelope (gp120) gene and the I3L-
promoted gag and pol genes were then inserted into a
vaccinia virus insertion vector. This was accomplished by
cloning the 6,400 bp NotI-BamHI fragment of pHIVGE15,
containing the H6-promoted gp120 gene and the I3L-promoted
gag and pol genes, into the 4,000 bp NotI-BglII fragment of
pSD542VCVQ. The plasmid generated by this manipulation is
called pHIVGE16.

pHIVGE16 was used in *in vitro* recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP988.

Immunoprecipitation experiments with vP988 infected
cells were performed as described above for the expression
of the HIV-1 gag precursor protein. No HIV-1-specific
species were precipitated from mock infected or NYVAC
infected Vero cells. Protein species corresponding to the
env and gag precursor proteins, as well as various
intermediate and mature gag cleavage products, were
precipitated, however, from lysates of vP988 infected cells.

**Example 98 - GENERATION OF NYVAC/HIV-1 gag/pol AND
               env (gp160) RECOMBINANT**

The sequence encoding the 5'-end of the gag gene was
cloned between vaccinia virus tk flanking arms. This was
accomplished by initially preparing plasmid pHIVGE16 as
described above (see Example 97).

The gp120 gene was then replaced by the gp160 gene.

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This was accomplished by cloning the 2,600 bp NruI-NotI
fragment of pH6HMNE, containing the entire HIV-1(MN)
envelope (gp160) gene, into the 8,000 bp partial NruI-NotI
fragment of pHIVGE16. The plasmid generated by this
manipulation is called pHIVGE19.

pHIVGE19 was used in in vitro recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP1009.

Immunoprecipitation experiments with vP1009 infected
cells were performed as described above for the expression
of the HIV-1 gag precursor protein. No HIV-1-specific
species were precipitated from mock infected or NYVAC
infected Vero cells. Protein species corresponding to the
env and gag precursor proteins, as well as various
intermediate and mature gag cleavage products, were
precipitated, however, from lysates of vP1009 infected
cells.

**Example 99   -   GENERATION OF ALVAC/HIV-1 gag/pol AND
                  env (GP120) RECOMBINANT**

The sequence encoding the 5'-end of the gag gene was
cloned between vaccinia virus tk flanking arms. This was
accomplished by intially preparing plasmid pHIVGE15, as
described above (see Example 97).

pHIVGE15 was used in recombination experiments with
CPpp (ALVAC) as the rescuing virus to yield vCP117.

Immunoprecipitation analysis was performed to determine
whether vCP117 expresses authentic HIV-1 gag and env gene
products. CEF cell monolayers were either mock infected,
infected with the parental virus or infected with vCP117 at
an m.o.i. of 10 PFU/cell. Following an hour adsorption
period, the inoculum was aspirated and the cells were
overlayed with 2 mls of modified Eagle's medium (minus
methionine) containing 2% fetal bovine serum and [^{35}S]-
methionine (20 μCi/ml). Cells were harvested at 18 hrs
post-infection by the addition of 1 ml 3X buffer A (3% NP-
40, 30mM Tris (pH7.4), 3mM EDTA, 0.03% Na Azide and 0.6
mg/ml PMSF) with subsequent scraping of the cell monolayers.

Lysates from the infected cells were analyzed for HIV
gag and env gene expression using serum from HIV-1
seropositive individuals (obtained from New York State

Department of Health). The sera was preadsorbed with CPpp
infected CEF cells. The preadsorbed sera was bound to
Protein A-sepharose in an overnight incubation at 4°C.
Following this incubation period, the material was washed 4X
with 1X buffer A. Lysates precleared with normal human sera
and protein A-sepharose were then incubated overnight at 4°C
with the HIV-1 seropositive human sera bound to protein A-
sepharose. After the overnight incubation period, the
samples were washed 4X with 1X buffer A and 2X with a
LiCl$_2$/urea buffer. Precipitated proteins were dissociated
from the immune complexes by the addition of 2X Laemmli's
buffer (125 mM Tris (pH6.8), 4% SDS, 20% glycerol, 10% 2-
mercaptoethanol) and boiling for 5 min. Proteins were
fractionated on a 10% Dreyfuss gel system (Dreyfuss et al.,
1984), fixed and treated with 1M Na-salicylate for
fluorography.

Human sera from HIV-1 seropositive individuals
specifically precipitated the HIV-1 _gag_ and env proteins
from vCP117 infected cells, but did not precipitate HIV-1-
specific proteins from mock infected or CPpp infected cells.

Example 100 - **GENERATION OF ALVAC/HIV-1 gag/pol AND**
                  env (gp160) RECOMBINANT

The sequence encoding the 5'-end of the _gag_ gene was
cloned between vaccinia virus tk flanking arms. This was
accomplished by initially preparing plasmid pHIVGE15 as
described above (_see_ Example 97).

The gp120 gene was then replaced by the gp160 gene.
This was accomplished by cloning the 2,600 bp _Nru_I-_Not_I
fragment of pH6HMNE, containing the entire HIV-1(MN)
envelope (gp160) gene, into the 9,800 bp _Nru_I-_Not_I fragment
of pHIVGE15. The plasmid generated by this manipulation is
called pHIVGE18.

The canary pox flanking arm deleted in the previous
step was then cloned into pHIVGE18. This was accomplished
by cloning the 1,500 bp _Not_I fragment of pHIVGE15,
containing the C3 flanking arm, into the 12,400 bp _Not_I
fragment of pHIVGE18. The plasmid generated by this
manipulation is called pHIVGE20.

pHIVGE20 was used in recombination experiments with

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CPpp (ALVAC) as the rescuing virus to yield vCP130.

Immunoprecipitation analysis was performed to determine whether vCP130 expresses authentic HIV-1 gag and env gene products. CEF cell monolayers were either mock infected, infected with the parental virus or infected with vCP130 at an m.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls of modified Eagle's medium (minus methionine) containing 2% fetal bovine serum and [^{35}S]-methionine (20 μCi/ml). Cells were harvested at 18 hrs post-infection by the addition of 1 ml 3X buffer A (3% NP-40, 30mM Tris (pH7.4), 3mM EDTA, 0.03% Na Azide and 0.6 mg/ml PMSF) with subsequent scraping of the cell monolayers.

Lysates from the infected cells were analyzed for HIV-1 gag and env gene expression using pooled serum from HIV-1 seropositive individuals (obtained from Chiron, Emeryville, CA). The sera was preadsorbed with CPpp infected CEF cells. The preadsorbed sera was bound to Protein A-sepharose in an overnight incubation at 4°C. Following this incubation period, the material was washed 4X with 1X buffer A. Lysates precleared with normal human sera and protein A-sepharose were then incubated overnight at 4°C with the HIV-1 seropositive human sera bound to protein A-sepharose. After the overnight incubation period, the samples were washed 4X with 1X buffer A and 2X with a LiCl$_2$/urea buffer. Precipitated proteins were dissociated from the immune complexes by the addition of 2X Laemmli's buffer (125 mM Tris (pH6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5 min. Proteins were fractionated on a 10% Dreyfuss gel system (Dreyfuss et al., 1984), fixed and treated with 1M Na-salicylate for fluorography. Human sera from HIV-1 seropositive individuals specifically precipitated the HIV-1 gag and env proteins from vCP130 infected cells, but did not precipitate HIV-1-specific proteins from mock infected or CPpp infected cells.

Example 101 - **GENERATION OF ALVAC/HIV-1 gag/pol RECOMBINANT**

A plasmid, pHXB2D, containing HIV-1 cDNA sequence was obtained from Dr. R.C. Gallo (NCI-NIH). The sequence

encoding the 5'-end of the gag gene was cloned between
vaccinia virus tk flanking arms. This was accomplished by
intially preparing plasmid pHIVG7 as described above (see
Example 89).

The gag and pol genes were then cloned between canary
pox flanking arms. This was accomplished by cloning the
4,400 bp SmaI-NotI fragment of pHIVG7, containing the I3L-
promoted gag and pol genes, and the oligonucleotides, HIV2L6
(SEQ ID NO:423) (5'-GGCCAAAC-3') and HIV2L7 (SEQ ID NO:424)
(5'-TCGAGTTT-3'), into the SmaI-XhoI site of pSPCP3L. The
plasmid generated by this manipulation is called pHIVG24.

pHIVG24 was used in recombination experiments with CPpp
(ALVAC) as the rescuing virus to yield vCP152.

Immunoprecipitation experiments with vCP152 infected
cells were performed as described above for the expression
of the HIV-1 env and gag proteins. No HIV-1-specific
species were precipitated from mock infected or ALVAC
infected cells. Protein species corresponding to the gag
precursor protein, as well as various intermediate and
mature gag cleavage products, were precipitated, however,
from lysates of vCP152 infected cells.

**Example 102 - GENERATION OF ALVAC/HIV-1 gag/pol AND
                 env (TRUNCATED) RECOMBINANT**

A plasmid, pHXB2D, containing HIV-1 cDNA sequence was
obtained from Dr. R.C. Gallo (NCI-NIH). The sequence
encoding the 5'-end of the gag gene was cloned between
vaccinia virus tk flanking arms. This was accomplished by
initially preparing plasmid pHIVG24 as described above (see
example 101).

pHIVG24 was used in recombination experiments with
vCP120 as the rescuing virus to yield vCP155.

Immunoprecipitation experiments with vCP155 infected
cells were performed as described above for the expression
of the HIV-1 env and gag proteins. No HIV-1-specific
species were precipitated from mock infected cells. Protein
species corresponding to the env and gag precursor proteins,
as well as various intermediate and mature gag cleavage
products, were precipitated, however, from lysates of vCP155
infected cells.

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**Example 103   -   GENERATION OF ALVAC/HIV-1 gag/pol AND env
                  (gp120 WITH TRANSMEMBRANE ANCHOR)
                  RECOMBINANT**

A plasmid, pHXB2D, containing HIV-1 cDNA sequence was
obtained from Dr. R.C. Gallo (NCI-NIH). The sequence
encoding the 5'-end of the gag gene was cloned between
vaccinia virus tk flanking arms. This was accomplished by
intially preparing plasmid pHIVG24 as described above (see
Example 101).

pHIVG24 was used in recombination experiments with
vCP138 as the rescuing virus to yield vCP156.

Immunoprecipitation experiments with vCP156 infected
cells were performed as described above for the expression
of the HIV-1 env and gag proteins. No HIV-1-specific
species were precipitated from mock infected cells. Protein
species corresponding to the env and gag precursor proteins,
as well as various intermediate and mature cleavage
products, were precipitated, however, from lysates of vCP156
infected cells.

Expression of HIV-1 gag-specific gene products either
alone or in combination with env by vaccinia virus has been
shown to lead to the production of non-infectious virus-like
particles (Haffar et al., 1990; Hu et al., 1990). With this
background we sought to investigate whether cells infected
ALVAC-based recombinant expressing HIV-1 gag-pol and env
genes would also produce such particles. Furthermore, if
these ALVAC-based recombinants were used to infect non-avian
cells (i.e. Vero, MRC-5, etc.) then HIV-1 virus-like
particles could be purified without any poxvirus virion
contaminants.

To evaluate particle formation using Vero cells
infected with vCP156, the following experiment was
performed. Vero cells were infected at an m.o.i. of
approximately 5 pfu/cell. After a 24 hr infection period,
the supernatant was harvested and clarified by
centrifugation at 2000 rpm for 10 min. The supernatant was
then spun through filters which have a molecular weight
cutoff of 30,000 kDa. Thus, any molecules smaller would
pass through these filters. The material retained by the
filters was then analyzed by standard Western blot analysis

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(Maniatis et al., 1990) using pooled human serum from HIV-
seropositive individuals (obtained from Dr. J. Conroy, New
York State Department of Health). The results from the
Western blot analysis demonstrated the presence of the major
core protein p24 and the HIV-1(MN) anchored gp120 in the
material retained by the filters. With the size exclusion
noted above, the p24 would have passed through unless it was
in a higher structural configuration (i.e. virus-like
particles). Therefore, these results strongly suggest that
HIV-1 virus-like particles containing the gp120 envelope
component are produced in vCP156 infected cells.

**Example 104 - EXPRESSION OF THE T1, T2, AND TH4.1 EPITOPES
                 OF THE HIV-1 env GENE IN ALVAC AND NYVAC**

     Recombinant poxviruses vP1062 and vCP146 were generated
to express the T1, T2, and TH4.1 epitopes of HIV-1 _env_
(Hosmalin et al., 1991) as individual peptides.

     Construction of plasmid p731T1. Plasmid pMPI3H
contains the vaccinia I3L early/intermediate promoter
element (Schmitt and Stunnenberg, 1988; Vos and Stunnenberg,
1988) in a pUC8 background. The promoter element was
synthesized by polymerase chain reaction (PCR) using pMPVC1,
a subclone of vaccinia HindIII I, as template and synthetic
oligonucleotides MPSYN283 (SEQ ID NO:425)
(5'- CCCCCCAAGCTTACATCATGCAGTGGTTAAAC -3') and MPSYN287 (SEQ
ID NO:426) (5'- GATTAAACCTAAATAATTGT -3') as primers. DNA
from this reaction was cut with HindIII and RsaI and a 0.1
kb fragment containing the promoter element was purified. A
linker region was assembled by annealing complementary
synthetic oligonucleotides MPSYN398 (SEQ ID NO:427)
(5'- ACAATTATTTAGGTTAACTGCA -3') and MPSYN399 (SEQ ID
NO:428) (5'- GTTAACCTAAATAATTGT -3'). The PCR-derived
promoter element and the polylinker region were ligated with
vector plasmid pUC8 which had been cut with HindIII and
PstI. The resulting plasmid, pMPI3H, contains the I3L
promoter region from positions -100 through -6 relative to
the initiation codon, followed by a polylinker region
containing HpaI, PstI, SalI, BamHI, SmaI and EcoRI sites.
Cleavage with HpaI produces blunt ended DNA linearized at
position -6 in the promoter.

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A cassette containing the T1 peptide driven by the
vaccinia I3L promoter was generated by ligating
complementary oligonucleotides T1C (SEQ ID NO:429)
(5'- TAATCATGAAACAAATTATTAATATGTGGCAAGAAGAGGAAAAGCTATGTACGCT
TGACTAGTTAATCACTCGAG -3') and T1N (SEQ ID NO:430)
(5'- GATCCTCGAGTGATTAACTAGTCAAGCGTACATAGCTTTTCCTACTTCTTGCCAC
ATATTAATAATTTGTTTCATGATTA -3') to plasmid pMPI3H digested
with HpaI and BamHI. This ligation reconstitutes the last 5
base pairs of the promoter, provides the complete coding
sequence of the T1 peptide, and creates a XhoI site between
the stop codon and BamHI site. This is plasmid p731T1. The
sequence of the fragment was confirmed by nucleotide
sequence analysis.

Construction of plasmid pH6T2. A cassette containing
the T2 peptide driven by the vaccinia H6 promoter was
generated in two steps: The H6 promoter through the EcoRV
site was derived from a plasmid containing the synthetic H6
promoter (Perkus et al., 1989), using PCR and primers H6PCR1
(SEQ ID NO:364) and H6PCR2 (SEQ ID NO:365), creating a 5'
HindIII site. This 122 bp PCR-derived fragment was digested
with HindIII and EcoRV followed by ligation to similarly
digested pBS-SK+ (Stratagene, La Jolla, CA), generating
plasmid pBSH6. Complementary oligonucleotides T2C (SEQ ID
NO:431) (5'- ATCCGTTAAGTTTGTATCGTAATGCACGAAGATATTATTTCTTTGT
GGGATCAATCTTTAAAATGACTAGTTAATCAG -3') and T2N (SEQ ID
NO:432) (5'- GATCCTGATTAACTAGTCATTTTAAAGATTGATCCCACAAAGAAATA
ATATCTTCGTGCATTACGATACAAACTTAACGGAT -3') which complete the
3' end of the H6 promoter from the EcoRV site, encode the T2
peptide and create a BamHI site at the 3' end of the gene
were annealed then ligated to pBSH6 that was digested with
EcoRV and BamHI. This plasmid was designated pH6T2
following confirmation of the fragment by nucleotide
sequence analysis.

Construction of plasmid pVQ42KTH4.1. A cassette
containing the TH4.1 peptide driven by the AmEPV 42K
promoter was generated by sequential PCR reactions: the 107
bp 42K promoter with 5' PstI and SmaI sites was derived by
PCR from plasmid p42KRABI, a plasmid containing the gene for
the rabies glycoprotein under control of the 42K promoter,

using primers 42KVQ1 (SEQ ID NO:433) (5'- AATTAATTAGCTGCAGCC
CCGGGTCAAAAAAATATAAATG -3') and 42KVQ2 (SEQ ID NO:434)
(5'- CCTTGTACTACTTCAATTACTCTATCCATTTTATATTGTAATTATATATTTTC).
The sequence of the 107 bp promoter region of this PCR-
derived fragment is (SEQ ID NO:435) 5'- TCAAAAAAATATAAATGATT
CACCATCTGATAGAAAAAAAATTTATTGGGAAGAATATGATAATATTTTGGGATTTCAAA
ATTGAAAATATATAATTACAATATAAA -3'. The 159 bp PCR-derived
fragment was fused to the coding sequences of TH4.1 with a
second PCR using this fragment and synthetic
oligonucleotides encoding the TH4.1 peptide TH41C (SEQ ID
NO:436) (5'- ATGGATAGAGTAATTGAAGTAGTACAAGGAGCTTATAGAGCTATTAG
ATGACTAGTTAATCACTCGAGGATCC -3') and TH41N (SEQ ID NO:437)
(5'- GGATCCTCGAGTGATTAACTAGTCATCTAATAGCTCTATAAGCTCCTTGTACTAC
TTCAATTACTCTATCCAT -3') as template and primers 42KTH41 (SEQ
ID NO:438) (5'- ATCATCGGATCCTCGAGTGATTAAACTAGTCATCTAATAGC
TC -3') and 42KVQ1 (SEQ ID NO:433). This 210 bp PCR-derived
fragment was extended in the 5' direction, incorporating a
BamHI site at the 5' end using the fragment and synthetic
oligonucleotides VQC (SEQ ID NO:439) (5'- TTAATCAGGATCCTT
AATTAATTAGTTATTAGACAAGGTGAAAACGAAACTATTTGTAGCTTAATTAATTAGCTG
CAGCCCGGG -3') and VQN (SEQ ID NO:440) (5'- CCCGGGCT
GCAGCTAATTAATTAAGCTACAAATAGTTTCGTTTTCACCTTGTCTAATAACTAATTAAT
TAAGGATCCTGATTAA -3') as template for a third PCR using
primers 42KTH41 (SEQ ID NO:438) and BAMVQ (SEQ ID NO:441)
(5'- TTAATCAGGATCCTTAATTAATTAGTTATTAGAC -3'). Subsequent
nucleotide sequence analysis revealed an error in the
sequence of oligonucleotide 42KTH41 (SEQ ID NO:438) such
that an extra base (an A) was inserted after position 24 as
indicated by the underline in the above sequence for
42KTH41. This was corrected with a final PCR employing the
272 bp fragment derived from the third PCR as template with
primers BAMVQ (SEQ ID NO:441) and 42KTH41A (SEQ ID NO:442)
(5'- ATCATCGGATCCTCGAGTGATTAACTAGTCATCTAATAGCTC -3'). After
the final PCR, the cassette was to contain BamHI, PstI, and
SmaI sites 5' to 42K-TH4.1 with XhoI and BamHI sites 3'.
This 271 bp PCR-derived fragment was digested with BamHI and
cloned into the BamHI site of pBS-SK+ (Stratagene, La Jolla,
CA) generating plasmid pVQ42KTH4.1. Nucleotide sequence
analysis of this plasmid confirmed that the sequence of the

promoter and coding region was correct. However, a 3 bp deletion was revealed resulting in loss of the 3' BamHI site.

Construction of plasmid pT1T2TH4.1. These three cassettes were combined into a singular plasmid pT1T2TH4.1 such that I3L-T1 is opposite in orientation to the other two genes in the following manner: A 170 bp HindIII/XhoI fragment was isolated from p731T1 and ligated to similarly digested pH6T2 generating pT1T2. A 290 bp BamHI/SacI fragment from pVQ42KTH4.1 was ligated to similarly digested pT1T2, creating pT1T2TH4.1. The sequence of the insert was confirmed by nucleotide sequence analysis.

pC5LSP (defined in Example 66) was digested with EcoRI, treated with alkaline phosphatase and ligated to self-annealed oligonucleotide CP29 (SEQ ID NO:363) (5'- AATTGCGGCCGC -3'), digested with NotI and linear purified followed by self-ligation. This procedure introduced a NotI site to pC5LSP, generating pNC5LSP5.

The 602 bp XhoI fragment from pT1T2TH4.1 containing the genes for the epitopes driven by their respective promoters was cloned into donor plasmids pNC5LSP5 and pSD550 (defined in Example 59) in their XhoI sites. Nucleotide sequence analysis was used to confirm the sequence and the orientation of the insert. The resulting plasmids pC5T1T2TH4.1 and pI4T1T2TH4.1 were used in in vitro recombination experiments with ALVAC and NYVAC to generate recombinant viruses vCP146 and vP1062, respectively. These recombinant viruses were demonstrated to contain the desired genes by hybridization of a specific DNA probe to viral plaques.

Example 105 - EXPRESSION OF TWO FUSION PEPTIDES CONTAINING THE T1, T2, AND TH4.1 EPITOPES OF HIV-1 env WITH AND WITHOUT A TRANSMEMBRANE ANCHOR DOMAIN FROM HIV-1 env

Recombinant poxviruses vP1060, vP1061, vCP154 and vCP148 were created to express a fusion peptide consisting of the signal sequences from HIV-1 env coupled to sequences corresponding to the T1, T2, and TH4.1 epitopes of HIV-1 env by cleavable linker regions. vP1060 and vCP154 differ from vP1061 and vCP148 in that the former recombinant viruses

express the fusion peptide along with sequences corresponding to the transmembrane region of HIV-1 env.

Both fusion peptides include the 51 amino acid N-terminal portion of HIV-1 (IIIB) env, residues 1-50 (plus initiating Met) based on Ratner et al. (1985). The amino acid sequence of this signal region (SEQ ID NO:443) is MKEQKTVAMRVKEKYQHLWRWGWRWGTMLLGMLMICSATEKLWVTVYYGVP. This is followed by the T1, T2, and TH4.1 epitopes (Hosmalin et al., 1991) separated from the signal, each other, and anchor sequence where present, by a cleavable linker region up to 5 amino acids in length. The amino acid sequence of this region of the peptide (SEQ ID NO:444) is [signal]-PFR-KQIINMWQEVGKAMYA-PPFRK-HEDIISLWDQSLK-PPFRK-DRVIEVVQGAYRAIR-[PPFRK-anchor]. The anchor domain is a 28 amino acid transmembrane region of HIV-1 (IIIB) env, residues 691-718. The amino acid sequence of this region (SEQ ID NO:445) is LFIMIVGGLVGLRIVFAVLSVVNRVRQG-[stop].

For both versions of the fusion peptide, the H6 promoter and HIV-1 env signal sequences were derived by PCR from plasmid pH6HIIIBEM (defined in Example 18) using primers H6PCR1 (SEQ ID NO:364) and PCRSIGT1 (SEQ ID NO:446) (5'- CATATTAATTTGTTTTCTAAAAGGAGGTACCCCATAATAGACTGTG -3'). This 314 bp PCR-derived fragment consists of a 5' HindIII site followed by the H6 promoter and coding sequences for the signal, linker, and the first 6 amino acids of the T1 peptide.

The remainder of the coding region for the construct without the transmembrane region was generated by PCR amplification of oligonucleotides T2T4A (SEQ ID NO:447) (5'- GCTCCTCCTTTTAGAAAACACGAAGATATTATTTCTTTGTGGGATCAATCTTTAA AACCTCCTTTTAGAAAAGATAGAGTAATTGAAGTAGTAC -3') and T2T4B (SEQ ID NO:448) (5'- GTACTACTTCAATTACTCTATCTTTTCTAAAAGGAGGTTTTA AAGATTGATCCCACAAAGAAATAATATCTTCGTGTTTTCTAAAAGGAGGAGC -3') using primers PCRT1T2 (SEQ ID NO:449) (5'- AAACAAATTA TTAATATGTGGCAAGAAGTAGGAAAAGCTATGTACGCTCCTCCTTTTAGAAAACACGAAG -3') and PCRT4END (SEQ ID NO:450) (5'- ACTACTTCTAGATTATCT AATAGCTCTATAAGCTCCTTGTACTACTTCAATTACTC -3'). This 177 bp PCR-derived fragment encodes the T1 peptide, a linker region, the T2 peptide, another linker region, and the TH4.1

peptide, followed by a 3' XbaI site. This fragment was
fused with the 314 bp PCR-derived fragment containing the
promoter and signal sequences by PCR with primers H6PCR1
(SEQ ID NO:364) and PCRT4END (SEQ ID NO:450). Following
digestion of this 473 bp PCR-derived fragment with HindIII
and XbaI a fragment of 455 bp was isolated from an agarose
gel, ligated to similarly digested pBS-SK, and the sequence
of the insert verified by nucleotide sequence analysis. The
resulting plasmid was designated pBST1T2TH4.1.

    The remainder of the coding region of the version with
the transmembrane anchor was generated by PCR amplification
of oligonucleotides, T2T4A (SEQ ID NO:447) and T2T4B (SEQ ID
NO:448), using primers PCRT1T2 (SEQ ID NO:449) and PCRT4TM
(SEQ ID NO:451) (5'- TACTATCATTATGAATAATTTTCTAAAAGGAGGTCTAAT
AGCTCTATAAGCTCCTTGTACTACTTCAATTACTC -3'), altering the 3'
end to accommodate the transmembrane region. This 195 bp
PCR-derived fragment was fused by PCR with oligonucleotides
comprising the anchor, HIVTM1 (SEQ ID NO:403) and HIVTM2
(SEQ ID NO:404) using primers PCRT1T2 (SEQ ID NO:449) and
PCRTMEND (SEQ ID NO:450). This 276 bp PCR-derived fragment
was fused with the 314 bp PCR-derived fragment containing
the promoter and signal sequences by PCR with primers H6PCR1
(SEQ ID NO:364) and PCRTMEND (SEQ ID NO:450). Following
digestion of this 572 bp PCR-derived fragment with HindIII
and XbaI a fragment of 554 bp was isolated from an agarose
gel, ligated to similarly digested pBS, and the sequence of
the insert verified by nucleotide sequence analysis. The
resulting plasmid was designated pBST1T2TH4.1A.

    pC5LSP (defined in Example 66) was digested with BamHI
and ligated to annealed oligonucleotides CP32 (SEQ ID
NO:406) and CP33 (SEQ ID NO:407) to generate pVQC5LSP6.
pVQC5LSP6 was digested with EcoRI, treated with alkaline
phosphatase and ligated to self-annealed oligonucleotide
CP29 (SEQ ID NO:363), digested with NotI and linear purified
followed by self-ligation. This procedure introduced a NotI
site to pVQC5LSP6, generating pNVQC5LSP7.

    Both cassettes were placed individually between the
XhoI and XbaI sites of insertion plasmid pNVQC5LSP7. These
plasmids pC5ST1T1TH4.1 and pC5ST1T2TH4.1A were used to

generate canarypoxviruses recombinant in the C5 locus,
vCP148 and vCP154 respectively. BamHI-SmaI fragments were
excised from pC5ST1T1TH4.1 and pC5ST1T2TH4.1A and ligated to
similarly digested pSD550 (defined in Example 59) generating
plasmids pI4ST1T2TH4.1 and pI4ST1T1TH4.1A, respectively.
These plasmids were used in IVR with the I4 locus of NYVAC,
resulting in recombinants vP1061 and vP1060, respectively.
These recombinant viruses were demonstrated to contain the
desired genes by hybridization of a specific DNA probe to
viral plaques.

Example 106 - EXPRESSION OF THE HIV-1 _nef_ GENE IN ALVAC,
**   TROVAC, AND NYVAC**

     Recombinant poxviruses vP1084, vFP174, and vCP168 were
generated expressing HIV-1 _nef_ (MN) as follows:

     The I3L promoter was derived by PCR from plasmid pMPI3H
using primers I3PCR1 (SEQ ID NO:452) (5'- ATCATCGGATCCAAGC
TTACATCATGCAGTGG -3') and PI3NEF2 (SEQ ID NO:453)
(5'- CGTTTTGACCATTTGCCACCCATGATTAAACCTAAATAATTGTACTTTG -3').
The coding region of _nef_ was generated by PCR amplification
of pMN1.8-10 (a clone of that portion of the MN genome
between SstI sites at 7792 and 9595) using primers PI3NEF1
(SEQ ID NO:454) (5'- AGTACAATTATTTAGGTTTAATCATGGGTGGCAAAT
GGTCAAAACG -3') and PNEFBAM (SEQ ID NO:455) (5'- ATCAT
CGGATCCTAACACTTCTCTCTCCGG -3'). Fusion of the coding region
with the promoter was accomplished by amplification of the
previous PCR products using primers I3PCR1 (SEQ ID NO:452)
and PNEFBAM (SEQ ID NO:455). Following digestion of this
product with BamHI a fragment of 0.7 kb was isolated from an
agarose gel and ligated to similarly digested pBS-SK+
(Stratagene, La Jolla, CA), generating plasmid pI3NEF. It
was at this point that sequence deviations were first
observed. The sequence of the cassette was determined to
differ from the published sequence (Gurgo et al., 1988,
GenBank Accession Number M17449) due to deviations in
plasmid pMN1.8-10. These differences are summarized below
relative to the published sequence.

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Base #	Gurgo et al.	pMN1.8-10	Result	Cassette
8834	T	T	silent	C (aa=Arg)
8863	G	A	Lys->Arg	A (aa=Lys)
8890	T	C	Pro->Leu	C (aa=Pro)
9028	A	G	Arg->Lys	G (aa=Arg)
9127	A	A	silent	G (aa=Gln)
9330-9331	GG	GGG	frameshift	GG

The two silent mutations in the cassette (at positions 8834
and 9127) were apparently errors in PCR. Since there is no
effect on the encoded protein, these were allowed to
persist. The frameshift at 9930 results in a lengthened
open reading frame more closely resembling other HIV-1
isolates. In keeping with the published size of *nef* from
the MN isolate, this cassette required a fourth PCR to
generate the truncated 3' end of the coding region.

Removal of the extra base at position 9930 was
accomplished by PCR amplification of the insert in pI3NEF
with primers I3PCR1 (SEQ ID NO:452) and PNEFFIX1 (SEQ ID
NO:456) (5'- ATCATCGGATCCTAACACTTCTCTCTCCGGGTCATCCATCCAT
GCTGGCTCATAG -3'). Following digestion of this 678 bp PCR-
derived fragment with <u>Bam</u>HI a fragment of 660 bp was
isolated from an agarose gel and ligated to similarly
digested pBS, generating plasmid pBSI3NEF. The insert was
verified by nucleotide sequence analysis.

The 660 bp <u>Bam</u>HI fragment from pBSI3NEF containing the
nef gene was placed in the <u>Bam</u>HI site of insertion plasmid
pNVQC5LSP7 (defined in Example 105). The resulting plasmid
pC5I3NEF was employed in an IVR into the C5 locus of ALVAC,
generating the recombinant vCP168. The same 660 bp <u>Bam</u>HI
fragment was also placed in the <u>Bam</u>HI site of insertion
plasmid pSD550VC (defined in Example 59) creating plasmid
pI4I3NEF. An IVR with this plasmid with NYVAC generated
recombinant vP1084.

An insertion plasmid for the F16 locos of TROVAC was
derived in the following manner. A 7.3 kb <u>Nae</u>I/<u>Nde</u>I
fragment was isolated from a plasmid containing the 10.5 kb
<u>Hind</u>III fragment of fowlpox virus described by Tartaglia et
al. (1990) and ligated to similarly digested pUC9 creating
plasmid pRW866.

pUC9 was digested with <u>Pvu</u>II and an <u>Eco</u>RI linker

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ligated between the PvuII sites creating plasmid pRW715. A
cloning site flanked by fowlpox sequences was generated by
PCR amplification of a portion of the 10.5 kb fragment with
primers RW264 (SEQ ID NO:457) (5'- AATTAACCCGGGATCCAAG
CTTCTAGCTAGCTAATTTTTATAGCGGCCGCTATAATCGTTAACTTATTAG -3') and
RW267 (SEQ ID NO:458) (5'- GCTAGAAATCTCTTAGTTTTTATA
GTTG -3'). An adjacent region was also amplified by PCR
using primers RW266 (SEQ ID NO:459) (5'- GTTACATATGTACA
GAATCTGATCATAG -3') and RW265 (SEQ ID NO:460)
(5'- CTAGCTAGAAGCTTGGATCCCGGGTTAATTAATTAATAAAAAGCGGCCGCGTTAA
AGTAGAAAAATG -3'). These PCR-derived fragments were fused
by a third PCR using primers RW266 (SEQ ID NO:459) and RW267
(SEQ ID NO:458). The resulting PCR-derived fragment
consisted of fowlpox sequences flanked by a 5' EcoRI site
and a 3' NdeI site. Central in the fragment is a
polycloning region containing SmaI, BamHI, and HindIII
sites, flanked by NotI sites and translation stop codons in
six reading frames. An early transcription termination
signal (Yuen and Moss, 1987) is adjacent to the 3' NotI
site. This PCR-derived fragment, digested with EcoRI and
NdeI, was ligated to similarly digested pRW715 creating
plasmid pRW864. An 11K promoted lac Z gene was excised from
pAM1 by partial BamHI, total PstI digestion. This fragment
was made blunt ended with Klenow polymerase and ligated to
SmaI digested pRW864, creating pRW867A. The NotI fragment
from pRW867A was made blunt ended with Klenow polymerase in
the presence of dNTPs so that the NotI sites would be
regenerated when ligated into an FspI site, and ligated to
pRW866 which was partially digested with FspI such that the
insertion was made corresponding to position 1955 described
by Tartaglia et al. (1990). The resulting plasmid, pRW868,
was then digested with NotI to remove the lac Z cassette,
and ligated to the 66 bp polylinker from pRW864 which was
excised by NotI digestion. The resulting plasmid was
designated pRW673. An 81 bp SmaI fragment was derived from
pVQ42KTH4.1 (defined in Example 104) and inserted into SmaI
digested pRW873 generating plasmid pVQ873.

        The nef cassette was excised from pBSI3NEF as a 684 bp
HindIII fragment for insertion into pVQ873 followed by IVR

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into the F16 locus of TROVAC to generate vFP174.

Example 107 - **EXPRESSION OF HIV-2 GENES IN NYVAC**

   Generation of NYVAC/HIV2 gag/pol recombinant.   A
plasmid, pISSYEGP, containing the human immunodeficiency
virus type 2 (HIV2) gag and pol genes was obtained from Dr.
G. Franchini (NCI-NIH). The gag and pol genes from this
plasmid were cloned downstream of the I3L promoter and
between vaccinia virus tk flanking arms. This was
accomplished by cloning a 4,400 bp BstUI-BglII fragment of
pISSYEGP, containing the HIV2 gag and pol genes, and the
oligonucleotides, SIVL1 (SEQ ID NO:181) and HIV2L1 (SEQ ID
NO:461) (5'-CGCCCATGATTAAACCTAAATAATTGTACTTTGTAATATAAT
GATATATATTTTCACTTTATCTCAC-3'), containing the I3L promoter,
into the 4,070 bp XhoI-BglII fragment of pSD542 (defined in
Example 32). The plasmid generated by this manipulation is
called pHIV21.

   Extraneous 3'-noncoding sequence was then eliminated.
This was accomplished by cloning a 280 bp BclI-SmaI PCR
fragment, containing the 3'-end of the pol gene, into the
8,100 bp BclI-SmaI fragment of pHIV21. This PCR fragment
was generated from the plasmid, pISSYEGP, with the
oligonucleotides, HIV2P2 (SEQ ID NO:462) (5'-
ATGGCAGTTCATTGCAT-3') and HIV2P3 (SEQ ID NO:463) (5'-
TTCCCGGGAGATCTCTATGCCATTTCTCCAT-3'). The plasmid generated
by this manipulation is called pHIV22.

   pHIV22 was used in recombination experiments with vP866
(NYVAC) as the rescuing virus to yield vP1045.

   Immunoprecipitation analysis was performed to determine
whether vP1045 expresses authentic HIV2 gag gene products.
Vero cell monolayers were either mock infected, infected
with the parental virus or infected with vP1045 at an m.o.i.
of 10 PFU/cell. Following an hour adsorption period, the
inoculum was aspirated and the cells were overlayed with 2
mls of modified Eagle's medium (minus methionine) containing
2% fetal bovine serum and [^{35}S]-methionine (20 μCi/ml).
Cells were harvested at 18 hrs post-infection by the
addition of 1 ml 3X buffer A (3% NP-40, 30mM Tris (pH7.4),
3mM EDTA, 0.03% Na Azide and 0.6 mg/ml PMSF) with subsequent
scraping of the cell monolayers.

     Lysates from the infected cells were analyzed for HIV2
gag expression using pooled serum from HIV2 seropositive
individuals (obtained from Dr. G. Franchini (NCI-NIH), NCI-
NIH, Bethesda, MD). The sera was preadsorbed with vP866
infected Vero cells. The preadsorbed sera was bound to
Protein A-sepharose in an overnight incubation at 4°C.
Following this incubation period, the material was washed 4X
with 1X buffer A. Lysates precleared with normal human sera
and protein A-sepharose were then incubated overnight at 4°C
with the HIV2 seropositive human sera bound to protein A-
sepharose. After the overnight incubation period, the
samples were washed 4X with 1X buffer A and 2X with a
$LiCl_2$/urea buffer. Precipitated proteins were dissociated
from the immune complexes by the addition of 2X Laemmli's
buffer (125 mM Tris (pH6.8), 4% SDS, 20% glycerol, 10% 2-
mercaptoethanol) and boiling for 5 min. Proteins were
fractionated on a 10% Dreyfuss gel system (Dreyfuss et al.,
1984), fixed and treated with 1M Na-salicylate for
fluorography.

     Human sera from HIV2 seropositive individuals
specifically precipitated the HIV2 _gag_ precursor protein, as
well as various intermediate and mature _gag_ cleavage protein
products, from vP1045 infected cells, but did not
precipitate HIV2-specific proteins from mock infected or
NYVAC infected cells.

**Example 108 - GENERATION OF NYVAC/HIV2 gag/pol AND
                 env (gp160) RECOMBINANT**

     A plasmid, pISSYEGP, containing the HIV2 _gag_ and _pol_
genes was obtained from Dr. G. Franchini (NCI-NIH). The gag
and _pol_ genes from this plasmid were cloned downstream of
the I3L promoter and between vaccinia virus tk flanking
arms. This was accomplished by initially preparing plasmid
pHIV22 as described above (_see_ Example 107).

     pHIV22 was used in recombination experiments with vP920
as the rescuing virus to yield vP1047.

     Immunoprecipitation experiments with vP1047 infected
cells were performed as described above for the expression
of the HIV2 _gag_ proteins. No HIV2-specific species were
precipitated from mock infected cells. Protein species

corresponding to the HIV2 env and gag precursor proteins, as
well as various intermediate and mature gag cleavage
products, were precipitated, however, from lysates of vP1047
infected cells.

**Example 109 - GENERATION OF NYVAC/HIV2 gag/pol AND
                 env (gp120) RECOMBINANT**

     A plasmid, pISSYEGP, containing the HIV2 gag and pol
genes was obtained from Dr. G. Franchini (NCI-NIH). The gag
and pol genes from this plasmid were cloned downstream of
the I3L promoter and between vaccinia virus tk flanking
arms. This was accomplished by initially preparing plasmid
pHIV22 as described above (see Example 107).

     pHIV22 was used in recombination experiments with vP922
as the rescuing virus to yield vP1044.

     Immunoprecipitation experiments with vP1044 infected
cells were performed as described above for the expression
of the HIV2 gag proteins. No HIV2-specific species were
precipitated from mock infected cells. Protein species
corresponding to the HIV2 env and gag precursor proteins, as
well as various intermediate and mature gag cleavage
products, were precipitated, however, from lysates of vP1044
infected cells.

Example 110 - EXPRESSION OF HIV2 GENES IN ALVAC

     Generation of ALVAC/HIV2 gag/pol and env (gp160)
recombinant. The plasmid, pBSH6HIV2ENV, contains the H6-
promoted HIV2 env (gp160) gene. The H6-promoted env gene
from this plasmid was cloned between canary pox flanking
arms. This was accomplished by cloning the 2,700 bp XhoI-
SacII fragment of pBSH6HIV2ENV, containing the H6-promoted
env gene, and the oligonucleotides, HIV2L4 (SEQ ID NO:464)
(5'-GGTTG-3') and HIV2L5 (SEQ ID NO:465) (5'-AATTCAACCGC-
3'), into the XhoI-EcoRI site of pC6L. The plasmid
generated by this manipulation is called pHIV23.

     The HIV2 gag and pol genes were then cloned into
pHIV23. This was accomplished by cloning the 4,450 bp XmaI-
NotI fragment of pHIV22, containing the I3L-promoted HIV2
gag and pol genes, and the oligonucleotides, HIV2L6 (SEQ ID
NO:423) and HIV2L7 (SEQ ID NO:424), into the 7,000 bp XmaI-
XhoI fragment of pHIV23. The plasmid generated by this

manipulation is called pHIV25.

pHIV25 was used in recombination experiments with CPpp (ALVAC) as the rescuing virus to yield vCP153.

Immunoprecipitation analysis was performed to determine whether vCP153 expresses authentic HIV2 _gag_ and env gene products. CEF cell monolayers were either mock infected, infected with the parental virus or infected with vCP153 at an m.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls of modified Eagle's medium (minus methionine) containing 2% fetal bovine serum and $[^{35}S]$-methionine (20 μCi/ml). Cells were harvested at 18 hrs post-infection by the addition of 1 ml 3X buffer A (3% NP-40, 30mM Tris (pH7.4), 3mM EDTA, 0.03% Na Azide and 0.6 mg/ml PMSF) with subsequent scraping of the cell monolayers.

Lysates from the infected cells were analyzed for HIV2 _gag_ and env gene expression using pooled serum from HIV2 seropositive individuals (obtained from Dr. G. Franchini (NCI-NIH), NCI-NIH, Bethesda, MD). The sera was preadsorbed with CPpp infected CEF cells. The preadsorbed sera was bound to Protein A-sepharose in an overnight incubation at 4°C. Following this incubation period, the material was washed 4X with 1X buffer A. Lysates precleared with normal human sera and protein A-sepharose were then incubated overnight at 4°C with the HIV2 seropositive human sera bound to protein A-sepharose. After the overnight incubation period, the samples were washed 4X with 1X buffer A and 2X with a LiCl$_2$/urea buffer. Precipitated proteins were dissociated from the immune complexes by the addition of 2X Laemmli's buffer (125 mM Tris (pH6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5 min. Proteins were fractionated on a 10% Dreyfuss gel system (Dreyfuss et al., 1984), fixed and treated with 1M Na-salicylate for fluorography.

Human sera from HIV2 seropositive individuals specifically precipitated the HIV2 env and _gag_ precursor proteins, as well as various intermediate and mature _gag_ cleavage products, from vCP153 infected cells, but did not precipitate HIV2-specific proteins from mock infected or

CPpp infected cells.

<u>Example 111</u> - <u>EXPRESSION OF SIV GENES IN NYVAC GENERATION
                OF NYVAC/SIV env (gp120-gp28) AND gag
                (PROTEASE⁻) RECOMBINANT</u>

     Immunoprecipitation analysis was performed to determine
whether vP948 (defined in Example 21) expresses authentic
SIV env and <u>gag</u> precursor proteins. Vero cell monolayers
were either mock infected, infected with the parental virus
or infected with vP948 at an m.o.i. of 10 PFU/cell.
Following an hour adsorption period, the inoculum was
aspirated and the cells were overlayed with 2 mls of
modified Eagle's medium (minus methionine) containing 2%
fetal bovine serum and [^{35}S]-methionine (20 μCi/ml). Cells
were harvested at 18 hrs post-infection by the addition of 1
ml 3X buffer A (3% NP-40, 30mM Tris (pH7.4), 3mM EDTA, 0.03%
Na Azide and 0.6 mg/ml PMSF) with subsequent scraping of the
cell monolayers.

     Lysates from the infected cells were analyzed for SIV
env and <u>gag</u> precursor expression using serum from SIV
seropositive macaques (obtained from Dr. G. Franchini (NCI-
NIH), NCI-NIH, Bethesda, MD). The sera was preadsorbed with
vP866 (NYVAC) infected Vero cells. The preadsorbed sera was
bound to Protein A-sepharose in an overnight incubation at
4°C. Following this incubation period, the material was
washed 4X with 1X buffer A. Lysates precleared with normal
macaque sera and protein A-sepharose were then incubated
overnight at 4°C with the SIV seropositive macaque sera
bound to protein A-sepharose. After the overnight
incubation period, the samples were washed 4X with 1X buffer
A and 2X with a LiCl$_2$/urea buffer. Precipitated proteins
were dissociated from the immune complexes by the addition
of 2X Laemmli's buffer (125 mM Tris (pH6.8), 4% SDS, 20%
glycerol, 10% 2-mercaptoethanol) and boiling for 5 min.
Proteins were fractionated on a 10% Dreyfuss gel system
(Dreyfuss et al., 1984), fixed and treated with 1M Na-
salicylate for fluorography.

     Macaque sera from SIV seropositive individuals
specifically precipitated the SIV <u>gag</u> precursor protein and
the envelope glycoprotein from vP948 infected cells, but did
not precipitate SIV-specific proteins from mock infected

cells.

Example 112   -   GENERATION OF NYVAC/SIV gag/pol RECOMBINANT

A plasmid, pSIVGAGSS11G, containing SIV_{MAC142} cDNA
sequence was obtained from Dr. G. Franchini (NCI-NIH). The
gag and pol genes from this plasmid were cloned downstream
of the I3L promoter and between vaccinia virus tk flanking
arms. This was accomplished by preparing plasmid pSIVG5 as
described above (see Example 21).

Extraneous 3'-noncoding sequence was then eliminated.
This was accomplished by cloning a 1,000 bp BamH1-HpaI PCR
fragment, containing the 3'-end of the pol gene into the
7,400 bp partial BamH1-HpaI fragment of pSIVG1. This PCR
fragment was generated from the plasmid, pSIVGAGSS11G, with
the oligonucleotides, SIVP5 (SEQ ID NO:183) and SIVP6 (SEQ
ID NO:184). The plasmid generated by this manipulation is
called pSIVG4.

Sequence analysis revealed that pSIVG4 contains a
single base pair deletion within the pol gene. To correct
this error the 2,320 bp BglII-StuI fragment of pSIVG1 was
cloned into the 6,100 bp partial BglII-StuI fragment of
pSIVG4. The plasmid generated by this manipulation is
called pSIVG5.

pSIVG5 was used in recombination experiments with vP866
(NYVAC) as the rescuing virus to yield vP1042.

Immunoprecipitation experiments with vP1042 infected
cells were performed as described above for the expression
of the SIV env and gag precursor proteins. No SIV-specific
species were precipitated from mock infected or NYVAC
infected Vero cells. Protein species corresponding to the
gag precursor protein, as well as various intermediate and
mature gag cleavage products, were precipitated, however,
from lysates of vP1042 infected cells.

**Example 113   -   GENERATION OF NYVAC/SIV gag/pol AND
                    env (gp120-gp41) RECOMBINANT**

pSIVG5 (Example 21) was used in recombination
experiments with vP1050 as the rescuing virus to yield
vP1071.

Immunoprecipitation experiments with vP1071 infected
cells show expression of SIV genes.

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**Example 114 - GENERATION OF NYVAC/SIV gag/pol AND
                   env (gp120-gp28) RECOMBINANT**

     pSIVG5 (Example 21) was used in recombination
experiments with vP874 as the rescuing virus to yield vP943.
     Immunoprecipitation experiments with vP943 infected
cells were performed as described above for the expression
of the SIV env and gag precursor proteins. No SIV-specific
species were precipitated from mock infected Vero cells.
Protein species corresponding to the env and gag precursor
proteins, as well as various intermediate and mature gag
cleavage products, were precipitated, however, from lysates
of vP943 infected cells.

Example 115 - GENERATION OF NYVAC/SIV p16, p28 RECOMBINANT

     Immunoprecipitation experiments with vP942 (Example 21)
infected cells were performed as described above for the
expression of the SIV env and gag precursor proteins. No
SIV-specific species were precipitated from mock infected
Vero cells. Protein species corresponding to p16 and p28
were precipitated, however, from lysates of vP942 infected
cells.

**Example 116 - GENERATION OF NYVAC/SIV p16, p28 AND
                   env (gp120-gp28) RECOMBINANT**

     Immunoprecipitation experiments with vP952 (Example 21)
infected cells were performed as described above for the
expression of the SIV env and gag precursor proteins. No
SIV-specific species were precipitated from mock infected
Vero cells. Protein species corresponding to env and p16
and p28 were precipitated, however, from lysates of vP952
infected cells.

**Example 117 - GENERATION OF NYVAC/SIV env (gp120-gp41)
                   RECOMBINANT**

     The plasmid, pSIVEMVC, contains the H6-promoted
SIV$_{MAC142}$ envelope gene (*in vitro* selected truncated
version). The region of the envelope gene containing the
premature termination codon was cloned into pBSK+. This was
accomplished by cloning the 1,120 bp ClaI-BamHI fragment of
pSIVEMVC into the ClaI-BamHI site of pBSK+. The plasmid
generated by this manipulation is called pSIV10.
     The upstream termination codon, TAG, was then changed

to the original CAG codon. This was accomplished by cloning
the oligonucleotides, SIVL20 (SEQ ID NO:466) (5'-CTAGCT
AAGTTAAGGCAGGGGTATAGGCCAGTGTTCTCTTCCCCACCCTCTTATTTCCAGCAGACT
CATACCCAACAG-3') and SIVL21 (SEQ ID NO:467) (5'- GTCCTGTT
GGGTATGAGTCTGCTGGAAATAAGAGGGTGGGGAAGAGAACACTGGCCTATACCCCTGCC
TTAACTTAG-3'), into the 4,000 bp <u>Nhe</u>I-<u>Ppu</u>MI fragment of
pSIV10. The plasmid generated by this manipulation is
called pSIV11.

     The region containing the modified codon was then
cloned back into pSIVEMVC. This was accomplished by cloning
the 380 bp <u>Bgl</u>II-<u>Nhe</u>I fragment of pSIV11, containing the
modified codon, into the 5,600 bp partial <u>Bgl</u>II-<u>Nhe</u>I
fragment of pSIVEMVC. The plasmid generated by this
manipulation is called pSIV12.

     pSIV12 was used in <i>in vitro</i> recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP1050.

     Immunoprecipitation experiments with vP1050 infected
cells were performed as described above for the expression
of the SIV env and <u>gag</u> precursor proteins. No SIV-specific
species were precipitated from mock infected or NYVAC
infected Vero cells. A protein species corresponding to env
was precipitated, however, from lysates of vP1050 infected
cells.

Example 118   -   EXPRESSION OF SIV GENES IN ALVAC

     <u>Generation of ALVAC/SIV gag/pol recombinant</u>. A
plasmid, pSIVGAGSS11G, containing SIV$_{MAC142}$ cDNA sequence was
obtained from Dr. G. Franchini (NCI-NIH). The <u>gag</u> and <u>pol</u>
genes from this plasmid were cloned downstream of the I3L
promoter and between vaccinia virus flanking arms. This was
accomplished by initially preparing plasmid pSIVG5 as
described above (<u>see</u> Example 21).

     The gag/pol genes were then cloned between canary pox
flanking arms. This was accomplished by cloning the 4,500
bp <u>Sma</u>I-<u>Not</u>I fragment of pSIVG5, containing the I3L-promoted
gag/pol genes, into the <u>Sma</u>I-<u>Not</u>I site of pC5L (defined in
Example 44). The plasmid generated by this manipulation is
called pSIVGC13.

     pSIVGC13 was used in recombination experiments with
CPpp (ALVAC as the rescuing virus to yield vCP172.

Immunoprecipitation experiments with vCP172 infected cells show expression of SIV genes.

Example 119 - **IMMUNIZATION OF HUMANS USING CANARYPOX EXPRESSING RABIES GLYCOPROTEIN (ALVAC-RG; vCP65)**

ALVAC-RG (VCP65) was generated as described in Example 15 and FIG. 17. For scaling-up and vaccine manufacturing ALVAC-RG (vCP65) was grown in primary CEF derived from specified pathogen free eggs. Cells were infected at a multiplicity of 0.01 and incubated at 37°C for three days.

The vaccine virus suspension was obtained by ultrasonic disruption in serum free medium of the infected cells; cell debris were then removed by centrifugation and filtration. The resulting clarified suspension was supplemented with lyophilization stabilizer (mixture of amino-acids), dispensed in single dose vials and freeze dried. Three batches of decreasing titer were prepared by ten-fold serial dilutions of the virus suspension in a mixture of serum free medium and lyophilization stabilizer, prior to lyophilization.

Quality control tests were applied to the cell substrates, media and virus seeds and final product with emphasis on the search for adventitious agents and innocuity in laboratory rodents. No desirable trait was found.

Preclinical data. Studies in vitro indicated that VERO or MRC-5 cells do not support the growth of ALVAC-RG (vCP65); a series of eight (VERO) and 10 (MRC) blind seria passages caused no detectable adaptation of the virus to grow in these non avian lines. Analyses of human cell lines (MRC-5, WISH Detroit 532, HEL, HNK or EBC transformed lymphoblastoid ALVAC-RG (vCP65)) showed no accumulation of virus specific DNA suggesting that in these cells the block in replication occurs prior to DNA synthesis. Significantly, however, the expression of the rabies virus glycoprotein gene in all cell lines tested indicating that the abortive step in the canarypox replication cycle occurs prior to viral DNA replication.

The safety and efficacy of ALVAC-RG (vCP65) were documented in a series of experiments in animals. A number of species including canaries, chickens, ducks, geese,

laboratory rodents (suckling and adult mice), hamsters,
guinea-pigs, rabbits, cats and dogs, squirrel monkeys,
rhesus macaques and chimpanzees, were inoculated with doses
ranging from 10^5 to 10^8 pfu. A variety of routes were used,
most commonly subcutaneous, intramuscular and intradermal
but also oral (monkeys and mice) and intracerebral (mice).

In canaries, ALVAC-RG (vCP65) caused a "take" lesion at
the site of scarification with no indication of disease or
death. Intradermal inoculation of rabbits resulted in a
typical poxvirus inoculation reaction which did not spread
and healed in seven to ten days. There was no adverse side
effects due to canarypox in any of the animal tests.
Immunogenicity was documented by the development of anti-
rabies antibodies following inoculation of ALVAC-RG (vCP65)
in rodents, dogs, cats, and primates, as measured by Rapid
Fluorescent Focus Inhibition Test (RFFIT). Protection was
also demonstrated by rabies virus challenge experiments in
mice, dogs, and cats immunized with ALVAC-RG (vCP65).

Volunteers. Twenty-five healthy adults aged 20-45 with
no previous history of rabies immunization were enrolled.
Their health status was assessed by complete medical
histories, physical examinations, hematological and blood
chemistry analyses. Exclusion criteria included pregnancy,
allergies, immune depression of any kind, chronic
debilitating disease, cancer, injection of immune globins in
the past three months, and seropositivity to human
immunodeficiency virus (HIV) or to hepatitis B virus surface
antigen.

Study design. Participants were randomly allocated to
receive either standard Human Diploid Cell Rabies Vaccine
(HDC) batch no E0751 (Pasteur Merieux Serums & Vaccine,
Lyon, France) or the study vaccine ALVAC-RG (vCP65).

The trial was designated as a dose escalation study.
Three batches of experimental ALVAC-RG (vCP65) vaccine were
used sequentially in three groups of volunteers (Groups A, B
and C) with two week intervals between each step. The
concentration of the three batches was $10^{3.5}$, $10^{4.5}$, $10^{5.5}$
Tissue Culture Infectious Dose ($TCID_{50}$) per dose,
respectively.

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Each volunteer received two doses of the same vaccine subcutaneously in the deltoid region at an interval of four weeks. The nature of the injected vaccine was not known by the participants at the time of the first injection but was known by the investigator.

In order to minimize the risk of immediate hypersensitivity at the time of the second injection, the volunteers of Group B allocated to the medium dose of experimental vaccine were injected 1 h previously with the lower dose and those allocated to the higher dose (Group C) received successively the lower and the medium dose at hourly intervals.

Six months later, the recipients of the highest dosage of ALVAC-RG (vCP65) (Group C) and HDC vaccine were offered a third dose of vaccine; they were then randomized to receive either the same vaccine as previously or the alternate vaccine. As a result, four groups were formed corresponding to the following immunization scheme: 1. HDC, HDC - HDC; 2. HDC, HDC - ALVAC-RG (vCP65); 3. ALVAC-RG (vCP65), ALVAC-RG (vCP65) - HDC; 4. ALVAC-RG (vCP65), ALVAC-RG (vCP65), ALVAC-RG (vCP65).

Monitoring of Side Effects. All subjects were monitored for 1 h after injection and re-examined every day for the next five days. They were asked to record local and systemic reactions for the next three weeks and were questioned by telephone two times a week.

Laboratory Investigators. Blood specimens were obtained before enrollment and two, four and six days after each injection. Analysis included complete blood cell count, liver enzymes and creatine kinase assays.

Antibody assays. Antibody assays were performed seven days prior to the first injection and at days 7, 28, 35, 56, 173, 187 and 208 of the study.

The levels of neutralizing antibodies to rabies were determined using the Rapid Fluorescent Focus Inhibition test (RFFIT) (Smith & Yaeger, In Laboratory Techniques on Rabies). Canarypox antibodies were measured by direct ELISA. The antigen, a suspension of purified canarypox virus disrupted with 0.1% Triton X100, was coated in

microplates. Fixed dilutions of the sera were reacted for
two hours at room temperature and reacting antibodies were
revealed with a peroxidase labelled anti-human IgG goat
serum. The results are expressed as the optical density
read at 490nm.

Analysis. Twenty-five subjects were enrolled and
completed the study. There were 10 males and 15 females and
the mean age was 31.9 (21 to 48). All but three subjects
had evidence of previous smallpox vaccination; the three
remaining subjects had no typical scar and vaccination
history. Three subjects received each of the lower doses of
experimental vaccine ($10^{3.5}$ and $10^{4.5}$ $TCID_{50}$), nine subjects
received $10^{5.5}$ $TCID_{50}$ and ten received the HDC vaccine.

Safety (Table 49). During the primary series of
immunization, fever greater than 37.7°C was noted within 24
hours after injection in one HDC recipient (37.8°C) and in
one vCP65 $10^{5.5}$ $TCID_{50}$ recipient (38°C). No other systemic
reaction attributable to vaccination was observed in any
participant.

Local reactions were noted in 9/10 recipients of HDC
vaccine injected subcutaneously and in 0/3, 1/3 and 9/9
recipients of vCP65 $10^{3.5}$, $10^{4.5}$, $10^{5.5}$ $TCID_{50}$, respectively.

Tenderness was the most common symptoms and was always
mild. Other local symptoms included redness and induration
which were also mild and transient. All symptoms usually
subsided within 24 hours and never lasted more than 72
hours.

There was no significant change in blood cell counts,
liver enzymes or creatine kinase values.

Immune Responses; Neutralizing Antibodies to Rabies
(Table 50). Twenty eight days after the first injection all
the HDC recipients had protective titers (≥0.5 IU/ml). By
contrast none in groups A and B ($10^{3.5}$ and $10^{4.5}$ $TCID_{50}$) and
only 2/9 in group C ($10^{5.5}$ $TCID_{50}$) ALVAC-RG (vCP65)
recipients reached this protective titer.

At day 56 (i.e. 28 days after the second injection)
protective titers were achieved in 0/3 of Group A, 2/3 of
Group B and 9/9 of Group C recipients of ALVAC-RG (vCP65)
vaccine and persisted in all 10 HDC recipients.

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At day 56 the geometric mean titers were 0.05, 0.47, 4.4 and 11.5 IU/ml in groups A, B, C and HDC respectively.

At day 180, the rabies antibody titers had substantially decreased in all subjects but remained above the minimum protective titer of 0.5 IU/ml in 5/10 HCD recipients and in 5/9 ALVAC-RG (vCP65) recipients; the geometric mean titers were 0.51 and 0.45 IU/ml in groups HCD and C, respectively.

Antibodies to the Canarypox virus (Table 51). The pre-immune titers observed varied widely with titers varying from 0.22 to 1.23 O.D. units despite the absence of any previous contact with canary birds in those subjects with the highest titers. When defined as a greater than two-fold increase between preimmunization and post second injection titers, a seroconversion was obtained in 1/3 subjects in group B and in 9/9 subjects in group C whereas no subject seroconverted in groups A or HDC.

Booster Injection. The vaccine was similarly well tolerated six months later, at the time of the booster injection: fever was noted in 2/9 HDC booster recipients and in 1/10 ALVAC-RG (vCP65) booster recipients. Local reactions were present in 5/9 recipients of HDC booster and in 6/10 recipients of the ALVAC-RG (vCP65) booster.

Observations. FIG. 35 shows graphs of rabies neutralizing antibody titers (Rapid Fluorescent Focus Inhibition Test or RFFIT, IU/ml): Booster effect of HDC and vCP65 ($10^{5.5}$ $TCID_{50}$) in volunteers previously immunized with either the same or the alternate vaccine. Vaccines were given at days 0, 28 and 180. Antibody titers were measured at days 0, 7, 28, 35, 56, 173, and 187 and 208.

As shown in FIG. 35 the booster dose given resulted in a further increase in rabies antibody titers in every subject whatever the immunization scheme. However, the ALVAC-RG (vCP65) booster globally elicited lower immune responses than the HDC booster and the ALVAC-RG (vCP65), ALVAC-RG (vCP65) - ALVAC-RG (vCP65) group had significantly lower titers than the three other groups. Similarly, the ALVAC-RG (vCP65) booster injection resulted in an increase in canarypox antibody titers in 3/5 subjects who had

previously received the HDC vaccine and in all five subjects
previously immunized with ALVAC-RG (vCP65).

In general, none of the local side effects from
administration of vCP65 was indicative of a local
replication of the virus. In particular, lesions of the
skin such as those observed after injection of vaccine were
absent. In spite of the apparent absence of replication of
the virus, the injection resulted in the volunteers
generating significant amounts of antibodies to both the
canarypox vector and to the expressed rabies glycoprotein.

Rabies neutralizing antibodies were assayed with the
Rapid Fluorescent Focus Inhibition Test (RFFIT) which is
known to correlate well with the sero neutralization test in
mice. Of 9 recipients of $10^{5.5}$ $TCID_{50}$, five had low level
responses after the first dose. Protective titers of rabies
antibodies were obtained after the second injection in all
recipients of the highest dose tested and even in 2 of the 3
recipients of the medium dose. In this study, both vaccines
were given subcutaneously as usually recommended for live
vaccines, but not for the inactivated HDC vaccine. This
route of injection was selected as it best allowed a careful
examination of the injection site, but this could explain
the late appearance of antibodies in HDC recipients: indeed,
none of the HDC recipients had an antibody increase at day
7, whereas, in most studies where HDC vaccine is give
intramuscularly a significant proportion of subjects do
(Klietmann et al., Int'l Green Cross - Geneva, 1981; Kuwert
et al., Int'l Green Cross - Geneva, 1981). However, this
invention is not necessarily limited to the subcutaneous
route of administration.

The GMT (geometric mean titers) of rabies neutralizing
antibodies was lower with the investigational vaccine than
with the HDC control vaccine, but still well above the
minimum titer required for protection. The clear dose
effect response obtained with the three dosages used in this
study suggest that a higher dosage might induce a stronger
response. Certainly from this disclosure the skilled
artisan can select an appropriate dosage for a given
patient.

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The ability to boost the antibody response is another important result of this Example; indeed, an increase in rabies antibody titers was obtained in every subject after the 6 month dose whatever the immunization scheme, showing that preexisting immunity elicited by either the canarypox vector or the rabies glycoprotein had no blocking effect on the booster with the recombinant vaccine candidate or the conventional HDC rabies vaccine. This contrasts findings of others with vaccinia recombinants in humans that immune response may be blocked by pre-existing immunity (Cooney et al, Lancet 1991, 337:567-72; Etlinger et al, Vaccine 1991, 9:470-72).

Thus, this Example clearly demonstrates that a non-replicating poxvirus can serve as an immunizing vector in humans, with all of the advantages that replicating agents confer on the immune response, but without the safety problem created by a fully permissive virus.

TABLE 49: Reactions in the 5 days following vaccination

vCP65 dosage (TCID50)	$10^{3.5}$		$10^{4.5}$		$10^{5.5}$		H D C control	
Injection	1st	2nd	1st	2nd	1st	2nd	1st	2nd
No. vaccinees	3	3	3	3	9	9	10	10
temp >37.7°C	0	0	0	0	0	1	1	0
soreness	0	0	1	1	6	8	8	6
redness	0	0	0	0	0	4	5	4
induration	0	0	0	0	0	4	5	4

TABLE 50: Rabies neutralizing antibodies (REFIT; IU/ml)
          Individual titers and geometric mean titers (GMT)

no.	TCID50/ dose	Days				
		0	7	28	35	56
1	$10^{3.5}$	< 0.1	< 0.1	< 0.1	< 0.1	0.2
3	$10^{3.5}$	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
4	$10^{3.5}$	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
	G.M.T.	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
6	$10^{4.5}$	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
7	$10^{4.5}$	< 0.1	< 0.1	< 0.1	2.4	1.9
10	$10^{4.5}$	< 0.1	< 0.1	< 0.1	1.6	1.1
	G.M.T.	< 0.1	< 0.1	0.1	0.58	0.47
11	$10^{5.5}$	< 0.1	< 0.1	1.0	3.2	4.3
13	$10^{5.5}$	< 0.1	< 0.1	0.3	6.0	8.8
14	$10^{5.5}$	< 0.1	< 0.1	0.2	2.1	9.4
17	$10^{5.5}$	< 0.1	< 0.1	< 0.1	1.2	2.5
18	$10^{5.5}$	< 0.1	< 0.1	0.7	8.3	12.5
20	$10^{5.5}$	< 0.1	< 0.1	< 0.1	0.3	3.7
21	$10^{5.5}$	< 0.1	< 0.1	0.2	2.6	3.9
23	$10^{5.5}$	< 0.1	< 0.1	< 0.1	1.7	4.2
25	$10^{5.5}$	< 0.1	< 0.1	< 0.1	0.6	0.9
	G.M.T.	< 0.1	< 0.1	0.16	1.9	4.4*
2	HDC	< 0.1	< 0.1	0.8	7.1	7.2
5	HDC	< 0.1	< 0.1	9.9	12.8	18.7
8	HDC	< 0.1	< 0.1	12.7	21.1	16.5
9	HDC	< 0.1	< 0.1	6.0	9.9	14.3
12	HDC	< 0.1	< 0.1	5.0	9.2	25.3
15	HDC	< 0.1	< 0.1	2.2	5.2	8.6
16	HDC	< 0.1	< 0.1	2.7	7.7	20.7
19	HDC	< 0.1	< 0.1	2.6	9.9	9.1
22	HDC	< 0.1	< 0.1	1.4	8.6	6.6
24	HDC	< 0.1	< 0.1	0.8	5.8	4.7
	G.M.T.	< 0.1	< 0.1	2.96	9.0	11.5*

* p = 0.007 student t test

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TABLE 51: Canarypox antibodies: ELISA Geometric Mean Titers*

vCP65 dosage TCID50/dose	Days				
	0	7	28	35	56
$10^{3.5}$	0.69	ND	0.76	ND	0.68
$10^{4.5}$	0.49	0.45	0.56	0.63	0.87
$10^{5.5}$	0.38	0.38	0.77	1.42	1.63
HDC control	0.45	0.39	0.40	0.35	0.39

* optical density at 1/25 dilution

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**Example 120   -   PROTECTION AGAINST JAPANESE ENCEPHALITIS
                   VIRUS BY NYVAC-JEV RECOMBINANTS
                   (vP908, vP923)**

Using NYVAC-JEV recombinants, protection against
Japanese Encephalitis virus was provided. NYVAC vP866,
NYVAC recombinants vP908 and vP923, and vaccinia
recombinants vP555 and vP829 were produced as described
herein.

Mouse Protection Experiments. Mouse protection
experiments were performed as previously described (Mason et
al., 1991). Briefly, groups of 10 to 12 4-week-old outbred
Swiss mice were immunized by intraperitoneal (i.p.)
injection with 10^7 PFU of vP829, vP866, vP908 or vP923, and
sera were collected three weeks alter. Mice were challenged
by i.p. injection with the Beijing P3 strain of JEV.
Following challenge, mice were observed at daily intervals
until the experiment was concluded three weeks later.
Lethal dose titrations were performed using litter mates of
the experimental animals.

Swine protection experiments. Landrace cross castrated
swine of about 25 kg body weight were used. Groups of five
pigs were immunized by subcutaneous (s.c.) injection with
phosphate buffered saline (PBS), or 1 x 10^8 PFU of vP866,
vP908 or vP923 diluted in 2 ml of PBS. Twenty-eight days
after inoculation, the pigs were boosted by injection in the
same manner as shown above, and 56 days after the first
inoculation they were challenged by s.c. injection with 2 x
10^5 PFU of the B-2358/84 strain of JEV. Sera were collected
from the animals in all groups on days 0 (before
immunization), 7, 14, 21, 28 (before boosting), 31, 35, 42,
56 (before challenge), 57, 58, 59, 60, 61, 62, 63, 64 and 76
(after challenge).

Evaluation of Immune Responses. Swine sera were tested
for their ability to precipitate JEV proteins from detergent
treated cell lysates or culture fluids obtained from
[^{35}S]Met-labeled JEV infected cells as described in Bonishi
et al. (1991), except for the use of fixed *Staphylococcus
aureus* bacteria coated with anti-swine immunoglobulin (Ig) G
(DAKO Corp., Carpinteria, California). The HAI test for
swine and mouse pre-challenge sera was performed by a

modification of the method of Clarke and Casals (1958). The
NEUT test was performed essentially as described by Mason et
al. (1991), except that freshly thawed human serum was not
used for testing pig sera.

   Viremia. Freshly thawed aliquots of spine sera
collected for eight days after JEV challenge were plaque
titrated for infectious JEV on VERO monolayer cells in 6-
well microplates. Following virus adsorption, the cell
monolayers were overlayed with medium containing 1%
carboxymethylcellulose, and plaques were visualized at five
days post-infection by staining with 0.1% crystal violet
dissolved in 20% ethanol. If three or fewer plaques were
observed in the wells incubated with 300 μl of serum, the
titer was recorded as <10 PFU/ml.

   Structures of the Recombinant Vaccinia Viruses. The
JEV cDNA sequences contained in vaccinia recombinants
constructed in this study are shown in FIG. 36. In these
recombinant viruses the sera strand of the JEV cDNA was
positioned behind the early/late H6 promoter. Recombinant
vP908 (and vP555; Mason et al., 1991) includes the putative
15 amino acid signal sequence preceding the N-terminus of
prM, prM, E, NS1 and NS2A. Recombinant vP923 (and vP828;
Konishi et al., 1991) encodes the putative signal sequence
of prM, prM, and E.

   Synthesis of E and NS1 by Recombinant Vaccinia Viruses.
Immunoprecipitation of the E or NS1 gene was performed using
a monoclonal antibody specific for E or NS1. Proteins
reactive with the E MAb were synthesized in cells infected
with vP555, vP908 and vP923, and proteins reactive with NS1
MAb were synthesized in cells infected with vP555 and vP908
but not in cells infected with vP923. vP555 infected cells
produced correct forms of E and NS1 inside and outside of
the cell. The proteins produced by vP908 and vP923 were
identical in size to those produced by vP555. For both E
and NS1, the extracellular forms migrated slower than the
intracellular forms in SDS-PAGE, consistent with maturation
of the N-linked glycans immediately preceding release from
the cells. In addition, vP908 infected cells produced a
higher molecular weight form of NS1, NS1', which is derived

by alternative processing of the sequences encoded by the
NS2A region of the JEV genome (Mason et al., 1987).
Immunoprecipitates prepared from radiolabeled vaccinia
recombinant infected cells using a MAb specific for M (and
prM) revealed that prM was synthesized in cells infected
with vP908 and vP923.

The extracellular fluid of vP908 or vP923 infected HeLa
cells showed an HA activity that was undetectable in the
culture fluid of vP866 infected cells. Data had indicated
that vP829 induced the synthesis of approximately eight
times more extracellular hemagglutinin than vP555 (Konishi
et al., 1991). In parallel to this difference, cells
infected with vP923 produced approximately 8 times more HA
activity than cells infected with vP908. These results
indicated that the recombinant form of JEV proteins produced
by vP980 and vP923 are equivalent to those produced by vP555
and vP829, respectively.

Immunization and Challenge of Mice. The ability of the
NYVAC based recombinant viruses to induce protective
immunity was examined in mice. The NEUT and HAI data for
the pre-challenge sera indicated that vP908 and vP923
produced the same levels of antibody as vP829 (Table 52) and
Konishi et al, 1991) (Table 52). Consistent with these
serological data, vP908 and vP923 were able to provide mice
with protection from lethal JEV infection by the pathogenic
P3 strain of JEV (Table 52). The levels of protection were
similar to that achieved by immunization with vP829 (Table
52) and Konishi et al., 1991). These studies confirmed that
vP908 and vP923, the two NYVAC based recombinants, were
immunogenic in mice at a level similar to those previously
described (Konishi et al., 1991).

Introduction of Immune Responses to JEV Antigens in
Swine by Recombinant Vaccinia Viruses. Pre-challenge sera
pooled from all swine in each group collected every seven
days were tested for anti-JEV NEUT and HAI activity. As
shown in FIG. 37, both NEUT and HAI activities were observed
in swine immunized with vP908 and vP923, but not in swine
inoculated with PBS or vP866. Relatively high levels of
NEUT antibodies were observed seven days after the first and

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second inoculation in both vP908 and vP923 immunized swine,
and decreased to barely detectable levels 21 days alter.
For both the primary inoculation and boost, HAI decreased
more slowly than NEUT antibodies (FIG. 37). vP908 provided
slightly higher levels of HAI and NEUT antibodies than
vP923. The HAI titers with both recombinants were greater
after the second inoculation than that achieved after a
single inoculation. vP923 provided a higher NEUT titer
after the second inoculation that upon a single inoculation,
whereas vP908 induced equivalent NEUT titers after a single
or double inoculation.

Swine were immunized on days 0 and 28, and sera
collected on day 56 from swine immunized with PBS or vP866
and on days 0, 7, 28, 35 and 56 from swine immunized with
vP908 or vP923. Sera pooled from all animals in each group
of 5 were tested for their ability to immunoprecipitate
radiolabeled proteins harvested from the culture fluid of
JEV-infected cells.

The immune response to E correlated well with the
results of the NEUT and HAI tests. The RIP response to E
observed in swine immunized with vP923 on day 35 was higher
than the RIP response to E in swine immunized with vP908,
whereas the HAI titers on day 35 were equivalent in the two
groups. However, NEUT titers on day 35 were higher in the
swine immunized with vP923 than in swine immunized with
vP908. Antibodies other than those involved in NEUT or HAI
may be induced but the quantitative aspects of the RIP
analysis was not further validated. Weak RIP responses of
sera to E on day seven in spite of relatively high NEUT
antibody titers could be explained by IgM antibody early
after immunization. Levels of JEV-specific IgM was not
further analyzed. None of the pooled sera showed an immune
response to NS1.

Viremia in Swine Immunized With Recombinant Vaccinia
Viruses. JE virus titers of >10 PFU/ml were detected in
post-challenge sera from the control swine [PBS (4/5) or
vP866 (5/5)], whereas only 2 of 5 swine inoculated with
vP923 exhibited viremias of >10 PFU/ml (FIG. 38).
Significantly, none of the five swine immunized with vP908

had measurable viremia >10 PFU/ml. The geometric mean of
the maximum virus titers in individual swine in groups
immunized with PBS and vP866 (1.2 x 10^3 PFU/ml) was
significantly higher than the mean in groups immunized with
vP908 and vP923 (1.9 x 10^1 PFU/ml; P<0.001 by the student t-
test; assuming a titer of 10 PFU/ml for all swine with
viremias of <10 PFU/ml). Moreover, the average period of
viremia for swine showing >10 PFU per ml of virus was 2.7
days for PBS and vP866, and 2.0 days for the two vP923
immunized swine which had titers of >10 PFU/ml. These
results indicate that immunization of vP908 and vP923
considerably reduced JEV viremia following challenge.

In order to evaluate the immune response to JEV
challenge, we tested individual sera collected 20 days post-
challenge for antibodies against JEV. The swine vaccinated
with vP908 or vP923 had higher responses to E than those
inoculated with PBS or vP866, indicating that the antibody
reactivity to E that was present before challenge was
boosted by JEV infection. Reactions to NS3 and NS5, JEV
proteins which were not expressed in the recombinant
vaccinia viruses were detected in all post-challenge sera
suggesting that some level of JEV replication occurred even
in pigs which had viremias of <10 PFU/ml. Thus, the
expected reciprocal relationship between reactivity of post-
challenge sera to NS3 and absence of infection as measured
by serum JEV titers was not observed. This is in contrast
to previous data obtained with mice in which lack of
reactivity of pooled mouse sera to NS3 post-challenge
correlated with protection (Konishi et al., 1991).

During the protection experiment, one pig in the
parental vaccinia (vP866) group died 12 days after the
challenge. This animal showed a higher body temperature
than any other swine during the post-challenge period and
was the only animal with JEV serum titers of >1,000 PFU for
three days (FIG. 38). Although JEV was not detected from
the brain specimen sampled at necropsy, the animal may have
died from the disease caused by JEV infection.

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Observations. The Example demonstrates that NYVAC-JEV provides effective protection against JEV viremia; and thus, NYVAC-JEV is useful and safe for veterinary applications.

Table 52. Immunization and JEV challenge in mice

Immunizing Virus[a]	JEV Genes Expressed	Antibody titer		Survival[d]
		NEUT[b]	HAI[c]	
vP829	prM, E	1:320	1:80	10/10 (100%)
vP866	None	<1:10	<1:10	0/12 (0%)
vP908	prM, E, NS	1:320	1:80	11/12 (92%)
vP923	prM, E	1:320	1:80	12/12 (100%)

[a]   Vaccinia recombinant virus used for immunizing groups of 4-week old mice.

[b]   Serum dilution yielding 90% reduction in plaque number (Mason et al., 1991)

[c]   Serum dilution.

[d]   Number survival/number challenged (% survival). Mice of each group were challenged with the Beijing P3 strain three weeks after immunization. The challenge doze was $3.8 \times 10^5 LD_{50}$ as determined by a lethal-dose titration using litter-mates of the mice.

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**Example 121   -   EVALUATION OF NYVAC (vP866) AND NYVAC-RG
                         (vP879)**

     Immunoprecipitations. Preformed monolayers of avian or
non-avian cells were inoculated with 10 pfu per cell of
parental NYVAC (vP866) or NYVAC-RG (vP879) virus. The
inoculation was performed in EMEM free of methionine and
supplemented with 2% dialyzed fetal bovine serum. After a
one hour incubation, the inoculum was removed and the medium
replaced with EMEM (methionine free) containing 20 μCi/ml of
^{35}S-methionine. After an overnight incubation of
approximately 16 hours, cells were lysed by the addition of
Buffer A (1% Nonidet P-40, 10 mM Tris pH7.4, 150 mM NaCl, 1
mM EDTA, 0.01% sodium azide, 500 units per ml of aprotinin,
and 0.02% phenyl methyl sulfonyl fluoride).
Immunoprecipitation was performed using a rabies
glycoprotein specific monoclonal antibody designated 24-3F10
supplied by Dr. C. Trinarchi, Griffith Laboratories, New
York State Department of Health, Albany, New York, and a rat
anti-mouse conjugate obtained from Boehringer Mannheim
Corporation (Cat. #605-500). Protein A Sepharose CL-48
obtained from Pharmacia LKB Biotechnology Inc., Piscataway,
New Jersey, was used as a support matrix.
Immunoprecipitates were fractionated on 10% polyacrylamide
gels according to the method of Dreyfuss et. al. (1984).
Gels were fixed, treated for fluorography with 1M Na-
salicylate for one hour, and exposed to Kodak XAR-2 film to
visualize the immunoprecipitated protein species.

     Sources of Animals. New Zealand White rabbits were
obtained from Hare-Marland (Hewitt, New Jersey). Three week
old male Swiss Webster outbred mice, timed pregnant female
Swiss Webster outbred mice, and four week old Swiss Webster
nude (nu$^+$nu$^+$) mice were obtained from Taconic Farms, Inc.
(Germantown, New York). All animals were maintained
according to NIH guidelines. All animal protocols were
approved by the institutional IACUC. When deemed necessary,
mice which were obviously terminally ill were euthanized.

     Evaluation of Lesions in Rabbits. Each of two rabbits
was inoculated intradermally at multiple sites with 0.1 ml
of PBS containing 10^4, 10^5, 10^6, 10^7, or 10^8 pfu of each test

virus or with PBS alone. The rabbits were observed daily
from day 4 until lesion resolution. Indurations and
ulcerations were measured and recorded.

Virus Recovery from Inoculation Sites. A single rabbit
was inoculated intradermally at multiple sites of 0/1 ml of
PBS containing 10^6, 10^7, or 10^8 pfu of each test virus or
with PBS alone. After 11 days, the rabbit was euthanized
and skin biopsy specimens taken from each of the inoculation
sites were aseptically prepared by mechanical disruption and
indirect sonication for virus recovery. Infectious virus
was assayed by plaque titration on CEF monolayers.

Virulence in Mice. Groups of ten mice, or five in the
nude mice experiment, were inoculated ip with one of several
dilutions of virus in 0.5 ml of sterile PBS. Reference is
also made to Example 24.

Cyclophosphamide (CY) Treatment. Mice were injected by
the ip route with 4 mg (0.02 ml) of CY (SIGMA) on day -2,
followed by virus injection on day 0. On the following days
post infection, mice were injected ip with CY: 4 mg on day
1; 2 mg on days 4, 7 and 11; 3 mg on days 14, 18, 21, 25 and
28. Immunosuppression was indirectly monitored by
enumerating white blood cells with a Coulter Counter on day
11. The average white blood cell count was 13,500 cells per
μl for untreated mice (n=4) and 4,220 cells per μl for CY-
treated control mice (n=5).

Calculation of LD_{50}. The lethal dose required to
produce 50% mortality (LD_{50}) was determined by the
proportional method of Reed and Muench (Reed and Muench
1938).

Potency Testing of NYVAC-RG in Mice. Four to six week
old mice were inoculated in the footpad with 50 to 100 μl of
a range of dilutions (2.0 - 8.0 $\log_{10}$ tissue culture
infective dose 50% ($TCID_{50}$)) of either VV-RG (Kieny et al.,
1984), ALVAC-RG (Taylor et al., 1991b), or the NYVAC-RG.
Each group consisted of eight mice. At 14 days post-
vaccination, the mice were challenged by intracranial
inoculation with 15 LD_{50} of the rabies virus CVS strain
(0.03 ml). On day 28, surviving mice were counted and
protective does 50% (PD_{50}) calculated.

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Derivation of NYVAC (vP866). The NYVAC strain of
vaccinia virus was generated from VC-2, a plaque cloned
isolate of the COPENHAGEN vaccine strain. To generate NYVAC
from VC-2, eighteen vaccinia ORFs, including a number of
viral functions associated with virulence, were precisely
deleted in a series of sequential manipulations as described
earlier in this disclosure. These deletions were
constructed in a manner designed to prevent the appearance
of novel unwanted open reading frames. FIG. 39
schematically depicts the ORFs deleted to generate NYVAC.
At the top of FIG. 39 is depicted the HindIII restriction
map of the vaccinia virus genome (VC-2 plaque isolate,
COPENHAGEN strain). Expanded are the six regions of VC-2
that were sequentially deleted in the generation of NYVAC.
The deletions were described earlier in this disclosure
(Examples 1 through 6). Below such deletion locus is listed
the ORFs which were deleted from that locus, along with the
functions or homologies and molecular weight of their gene
products.

Replication Studies of NYVAC and ALVAC on Human Tissue
Cell Lines. In order to determine the level of replication
of NYVAC strain of vaccinia virus (vP866) in cells of human
origin, six cell lines were inoculated at an input
multiplicity of 0.1 pfu per cell under liquid culture and
incubated for 72 hours. The COPENHAGEN parental clone (VC-
2) was inoculated in parallel. Primary chick embryo
fibroblast (CEF) cells (obtained from 10-11 day old
embryonated eggs of SPF origin, Spafas, Inc., Storrs, CT)
were included to represent a permissive cell substrate for
all viruses. Cultures were analyzed on the basis of two
criteria: the occurrence of productive viral replication
and expression of an extrinsic antigen.

The replication potential of NYVAC in a number of human
derived cells are shown in Table 53. Both VC-2 and NYVAC
are capable of productive replication in CEF cells, although
NYVAC with slightly reduced yields. VC-2 is also capable of
productive replication in the six human derived cell lines
tested with comparable yields except in the EBV transformed
lymphoblastoid cell line JT-1 (human lymphoblastoid cell

line transformed with Epstein-Barr virus, <u>see</u> Rickinson et
al., 1984). In contract, NYVAC is highly attenuated in its
ability to productively replicate in any of the human
derived cell lines tested. Small increases of infectious
virus above residual virus levels were obtained from NYVAC-
infected MRC-5 (ATCC #CCL171, human embryonic lung origin),
DETROIT 532 (ATCC #CCL54, human foreskin, Downs Syndrome),
HEL 299 (ATCC #CCL137, human embryonic lung cells) and HNK
(human neonatal kidney cells, Whittiker Bioproducts, Inc.
Walkersville, MD, Cat #70-151) cells. Replication on these
cell lines was significantly reduced when compared to virus
yields obtained from NYVAC-infected CEF cells or with
parental VC-2 (Table 53). It should be noted that the
yields at 24 hours in CEF cells for both NYVAC and VC-2 is
equivalent to the 72-hour yield. Allowing the human cell
line cultures to incubate an additional 48 hours (another
two viral growth cycles) may, therefore, have amplified the
relative virus yield obtained.

Consistent with the low levels of virus yields obtained
in the human-derived cell lines, MRC-5 and DETROIT 532,
detectable but reduced levels of NYVAC-specific DNA
accumulation were noted. The level of DNA accumulation in
the MRC-5 and DETROIT 532 NYVAC-infected cell lines relative
to that observed in NYVAC-infected CEF cells paralleled the
relative virus yields. NYVAC-specific viral DNA
accumulation was not observed in any of the other human-
derived cells.

An equivalent experiment was also performed using the
avipox virus, ALVAC. The results of virus replication are
also shown in Table 53. No progeny virus was detectable in
any of the human cell lines consistent with the host range
restriction of canarypox virus to avian species. Also
consistent with a lack of productive replication of ALVAC in
these human-derived cells is the observation that no ALVAC-
specific DNA accumulation was detectable in any of the
human-derived cell lines.

<u>Expression of Rabies Glycoprotein by NYVAC-RG (vP879)
in Human Cells</u>. In order to determine whether efficient
expression of a foreign gene could be obtained in the

absence of significant levels of productive viral
replication, the same cell lines were inoculated with the
NYVAC recombinant expressing the rabies virus glycoprotein
(vP879, Example 7) in the presence of ^{35}S-methionine.
Immunoprecipitation of the rabies glycoprotein was performed
from the radiolabelled culture lysate using a monoclonal
antibody specific for the rabies glycoprotein.
Immunoprecipitation of a 67kDa protein was detected
consistent with a fully glycosylated form of the rabies
glycoprotein. No serologically crossreactive product was
detected in uninfected or parental NYVAC infected cell
lysates. Equivalent results were obtained with all other
human cells analyzed.

Inoculations on the Rabbit Skin. The induction and
nature of skin lesions on rabbits following intradermal (id)
inoculations has been previously used as a measure of
pathogenicity of vaccinia virus strains (Buller et al.,
1988; Child et al., 1990; Fenner, 1958, Flexner et al.,
1987; Ghendon and Chernos 1964). Therefore, the nature of
lesions associated with id inoculations with the vaccinia
strains WR (ATCC #VR119 plaque purified on CV-1 cells, ATCC
#CCL70, and a plaque isolate designated L variant, ATCC
#VR2035 selected, as described in Panicali et al., 1981)),
WYETH (ATCC #VR325 marketed as DRYVAC by Wyeth Laboratories,
Marietta, PA), COPENHAGEN (VC-2), and NYVAC was evaluated by
inoculation of two rabbits (A069 and A128). The two rabbits
displayed different overall sensitivities to the viruses,
with rabbit A128 displaying less severe reactions than
rabbit A069. In rabbit A128, lesions were relatively small
and resolved by 27 days post-inoculation. On rabbit A069,
lesions were intense, especially for the WR inoculation
sites, and resolved only after 49 days. Intensity of the
lesions was also dependent on the location of the
inoculation sites relative to the lymph drainage network.
In particular, all sites located above the backspine
displayed more intense lesions and required longer times to
resolve the lesions located on the flanks. All lesions were
measured daily from day 4 to the disappearance of the last
lesion, and the means of maximum lesion size and days to

resolution were calculated (Table 54). No local reactions
were observed from sites injected with the control PBS.
Ulcerative lesions were observed at sites injected with WR,
VC-2 and WYETH vaccinia virus strains. Significantly, no
induration or ulcerative lesions were observed at sites of
inoculation with NYVAC.

Persistence of Infectious Virus at the Site of
Inoculation. To assess the relative persistence of these
viruses at the site of inoculation, a rabbit was inoculated
intradermally at multiple sites with 0.1 ml PBS containing
10^6, 10^7 or 10^8 pfu of VC-2, WR, WYETH or NYVAC. For each
virus, the 10^7 pfu dose was located above the backspine,
flanked by the 10^6 and 10^8 doses. Sites of inoculation were
observed daily for 11 days. WR elicited the most intense
response, followed by VC-2 and WYETH (Table 55). Ulceration
was first observed at day 9 for WR and WYETH and day 10 for
VC-2. Sites inoculated with NYVAC or control PBS displayed
no induration or ulceration. At day 11 after inoculation,
skin samples from the sites of inoculation were excised,
mechanically disrupted, and virus was titrated on CEF cells.
The results are shown in Table 55. In no case was more
virus recovered at this timepoint than was administered.
Recovery of vaccinia strain, WR, was approximately 10^6 pfu
of virus at each site irrespective of amount of virus
administered. Recovery of vaccinia strains WYETH and VC-2
was 10^3 to 10^4 pfu regardless of amount administered. No
infectious virus was recovered from sites inoculated with
NYVAC.

Inoculation of Genetically or Chemically Immune
Deficient Mice. Intraperitoneal inoculation of high doses
of NYVAC (5 X 10^8 pfu) or ALVAC (10^9 pfu) into nude mice
caused no deaths, no lesions, and no apparent disease
through the 100 day observation period. In contrast, mice
inoculated with WR (10^3 to 10^4 pfu), WYETH (5 x 10^7 or 5 x
10^8 pfu) or VC-2 (10^4 to 10^9 pfu) displayed disseminated
lesions typical of poxviruses first on the toes, then on the
tail, followed by severe orchitis in some animals. In mice
infected with WR or WYETH, the appearance of disseminated
lesions generally led to eventual death, whereas most mice

infected with VC-2 eventually recovered. Calculated LD_{50}
values are given in Table 56.

In particular, mice inoculated with VC-2 began to
display lesions on their toes (red papules) and 1 to 2 days
later on the tail. These lesions occurred between 11 and 13
days post-inoculation (pi) in mice given the highest doses
(10^9, 10^8, 10^7 and 10^6 pfu), on day 16 pi in mice given 10^5
pfu and on day 21 pi in mice given 10^4 pfu. No lesions were
observed in mice inoculated with 10^3 and 10^2 pfu during the
100 day observation period. Orchitis was noticed on day 23
pi in mice given 10^9 and 10^8 pfu, and approximately 7 days
later in the other groups (10^7 to 10^4 pfu). Orchitis was
especially intense in the 10^9 and 10^8 pfu groups and,
although receding, was observed until the end of the 100 day
observation period. Some pox-like lesions were noticed on
the skin of a few mice, occurring around 30-35 days pi.
Most pox lesions healed normally between 60-90 days pi.
Only one mouse died in the group inoculated with 10^9 pfu
(Day 34 pi) and one mouse died in the group inoculated with
10^8 pfu (Day 94 pi). No other deaths were observed in the
VC-2 inoculated mice.

Mice inoculated with 10^4 pfu of the WR strain of
vaccinia started to display pox lesions on Day 17 pi. These
lesions appeared identical to the lesions displayed by the
VC-2 injected mice (swollen toes, tail). Mice inoculated
with 10^3 pfu of the WR strain did not develop lesions until
34 days pi. Orchitis was noticed only in the mice
inoculated with the highest dose of WR (10^4 pfu). During
the latter stages of the observation period, lesions
appeared around the mouth and the mice stopped eating. All
mice inoculated with 10^4 pfu of WR died or were euthanized
when deemed necessary between 21 days and 31 days pi. Four
out of the 5 mice injected with 10^3 pfu of WR died or were
euthanized when deemed necessary between 35 days and 57 days
pi. No deaths were observed in mice inoculated with lower
doses of WR (1 to 100 pfu).

Mice inoculated with the WYETH strain of vaccinia virus
at higher doses 5 x 10^7 and 5 x 10^8 pfu) showed lesions on
toes and tails, developed orchitis, and died. Mice injected

with 5 x 10^6 pfu or less of WYETH showed no signs of disease or lesions.

As shown in Table 56, CY-treated mice provided a more sensitive model for assaying poxvirus virulence than did nude mice. LD_{50} values for the WR, WYETH, and VC-2 vaccinia virus strains were significantly lower in this model system than in the nude mouse model. Additionally, lesions developed in mice injected with WYETH, WR and VC-2 vaccinia viruses, as noted below, with higher doses of each virus resulting in more rapid formation of lesions. As was seen with nude mice, CY-treated mice injected with NYVAC or ALVAC did not develop lesions. However, unlike nude mice, some deaths were observed in CY-treated mice challenged with NYVAC or ALVAC, regardless of the dose. These random incidences are suspect as to the cause of death.

Mice injected with all doses of WYETH (9.5 x 10^4 to 9.5 x 10^8 pfu) displayed pox lesions on their tail and/or on their toes between 7 and 15 days pi. In addition, the tails and toes were swollen. Evolution of lesions on the tail was typical of pox lesions with formation of a papule, ulceration and finally formation of a scab. Mice inoculated with all doses of VC-2 (1.65 x 10^5 to 1.65 x 10^9) also developed pox lesions on their tails and/or their toes analogous to those of WYETH injected mice. These lesions were observed between 7-12 days post inoculation. No lesions were observed on mice injected with lower doses of WR virus, although deaths occurred in these groups.

Potency Testing of NYVAC-RG. In order to determine that attenuation of the COPENHAGEN strain of vaccinia virus had been effected without significantly altering the ability of the resulting NYVAC strain to be a useful vector, comparative potency tests were performed. In order to monitor the immunogenic potential of the vector during the sequential genetic manipulations performed to attenuate the virus, a rabiesvirus glycoprotein was used as a reporter extrinsic antigen. The protective efficacy of the vectors expressing the rabies glycoprotein gene was evaluated in the standard NIH mouse potency test for rabies (Seligmann, 1973). Table 57 demonstrates that the PD_{50} values obtained

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with the highly attenuated NYVAC vector are identical to
those obtained using a COPENHAGEN-based recombinant
containing the rabies glycoprotein gene in the _tk_ locus
(Kieny et al., 1984) and similar to PD_{50} values obtained
with ALVAC-RG, a canarypox based vector restricted to
replication to avian species.

    Observations. NYVAC, deleted of known virulence genes
and having restricted _in vitro_ growth characteristics, was
analyzed in animal model systems to assess its attenuation
characteristics. These studies were performed in comparison
with the neurovirulent vaccinia virus laboratory strain, WR,
two vaccinia virus vaccine strains, WYETH (New York City
Board of Health) and COPENHAGEN (VC-2), as well as with a
canarypox virus strain, ALVAC (_See also_ Example 24).
Together, these viruses provided a spectrum of relative
pathogenic potentials in the mouse challenge model and the
rabbit skin model, with WR being the most virulent strain,
WYETH and COPENHAGEN (VC-2) providing previously utilized
attenuated vaccine strains with documented characteristics,
and ALVAC providing an example of a poxvirus whose
replication is restricted to avian species. Results from
these _in vivo_ analyses clearly demonstrate the highly
attenuated properties of NYVAC relative to the vaccinia
virus strains, WR, WYETH and COPENHAGEN (VC-2) (Tables 28-
29, 53-57). Significantly, the LD_{50} values for NYVAC were
comparable to those observed with the avian host restricted
avipoxvirus, ALVAC. Deaths due to NYVAC, as well as ALVAC,
were observed only when extremely high doses of virus were
administered via the intracranial route (Example 24, Tables
28, 29, 56). It has not yet been established whether these
deaths were due to nonspecific consequences of inoculation
of a high protein mass. Results from analyses in
immunocompromised mouse models (nude and CY-treated) also
demonstrate the relatively high attenuation characteristics
of NYVAC, as compared to WR, WYETH and COPENHAGEN strains
(Tables 54 and 55). Significantly, no evidence of
disseminated vaccinia infection or vaccinial disease was
observed in NYVAC-inoculated animals or ALVAC-inoculated
animals over the observation period. The deletion of

multiple virulence-associated genes in NYVAC shows a
synergistic effect with respect to pathogenicity. Another
measure of the innocuity of NYVAC was provided by the
intradermal administration on rabbit skin (Tables 54 and
55). Considering the results with ALVAC, a virus unable to
replicate in nonavian species, the ability to replicate at
the site of inoculation is not the sole correlate with
reactivity, since intradermal inoculation of ALVAC caused
areas of induration in a dose dependent manner (unpublished
observations). Therefore, it is likely that factors other
than the replicative capacity of the virus contribute to the
formation of the lesions. Deletion of genes in NYVAC
prevents lesion occurrence.

Together, the results in this Example and in foregoing
Examples, including Example 24, demonstrate the highly
attenuated nature of NYVAC relative to WR, and the
previously utilized vaccinia virus vaccine strains, WYETH
and COPENHAGEN. In fact, the pathogenic profile of NYVAC,
in the animal model systems tested, was similar to that of
ALVAC, a poxvirus known to productively replicate only in
avian species. The apparently restricted capacity of NYVAC
to productively replicate on cells derived from humans
(Table 53) and other species, including the mouse, swine,
dog and horse, provides a considerable barrier that limits
or prevents potential transmission to unvaccinated contacts
or to the general environment in addition to providing a
vector with reduced probability of dissemination within the
vaccinated individual.

Significantly, NYVAC-based vaccine candidates have been
shown to be efficacious. NYVAC recombinants expressing
foreign gene products from a number of pathogens have
elicited immunological responses towards the foreign gene
products in several animal species, including primates. In
particular, a NYVAC-based recombinant expressing the rabies
glycoprotein was able to protect mice against a lethal
rabies challenge. The potency of the NYVAC-based rabies
glycoprotein recombinant was comparable to the PD_{50} value
for a COPENHAGEN-based recombinant containing the rabies
glycoprotein in the tk locus (Table 57). NYVAC-based

recombinants have also been shown to elicit measles virus
neutralizing antibodies in rabbits and protection against
pseudorabies virus and Japanese encephalitis virus challenge
in swine. The highly attenuated NYVAC strain confers safety
advantages with human and veterinary applications (Tartaglia
et al., 1990). Furthermore, the use of NYVAC as a general
laboratory expression vector system may greatly reduce the
biological hazards associated with using vaccinia virus.

     By the following criteria, the results of this Example
and the foregoing Examples, including Example 24, show NYVAC
to be highly attenuated: a) no detectable induration or
ulceration at site of inoculation (rabbit skin); b) rapid
clearance of infectious virus from intradermal site of
inoculation (rabbit skin); c) absence of testicular
inflammation (nude mice); d) greatly reduced virulence
(intracranial challenge, both three-week old and newborn
mice); e) greatly reduced pathogenicity and failure to
disseminate in immunodeficient subjects (nude and
cyclophosphamide treated mice); and f) dramatically reduced
ability to replicate on a variety of human tissue culture
cells. Yet, in spite of being highly attenuated, NYVAC, as
a vector, retains the ability to induce strong immune
responses to extrinsic antigens.

TABLE 53   Replication of COPENHAGEN (VC-2), NYVAC and
                ALVAC in avian or human derived cell lines

Cells	Hours post-infection	Yield[a]			% Yield
		VC-2	NYVAC	ALVAC	
CEF	0	3.8[b]	3.7	4.5	
	24	8.3	7.8	6.6	
	48	8.6	7.9	7.7	
	72	8.3	7.7	7.5	25
	72A[c]	<1.4	1.8	3.1	
MRC-5	0	3.8	3.8	4.7	
	72	7.2	4.6	3.8	0.25
	72A	2.2	2.2	3.7	
WISH*	0	3.4	3.4	4.3	
	72	7.6	2.2	3.1	0.0004
	72A	-[d]	1.9	2.9	
DETROIT	0	3.8	3.7	4.4	
	72	7.2	5.4	3.4	1.6
	72A	1.7	1.7	2.9	
HEL	0	3.8	3.5	4.3	
	72	7.5	4.6	3.3	0.125
	72A	2.5	2.1	3.6	
JT-1	0	3.1	3.1	4.1	
	72	6.5	3.1	4.2	0.039
	72A	2.4	2.1	4.4	
HNK	0	3.8	3.7	4.7	
	72	7.6	4.5	3.6	0.079
	72A	3.1	2.7	3.7	

a: Yield of NYVAC at 72 hours post-infection expressed as
    a percentage of yield of VAC-2 after 72 hours on the
    same cell line.
b: Titer expressed as LOG_{50} pfu per ml.
c: Sample was incubated in the presence of 40μg/ml of
    cytosine arabinoside.
d: Not determined.
*: ATCC #CCL25 Human amnionic cells.

Table 54.   Induration and ulceration at the site of
                 intradermal inoculation of the rabbit skin

VIRUS STRAIN	DOSE[a]	INDURATION		ULCERATION	
		Size[b]	Days[c]	Size	Days
WR	10^4	386	30	88	30
	10^5	622	35	149	32
	10^6	1057	34	271	34
	10^7	877	35	204	35
	10^8	581	25	88	26
WYETH	10^4	32	5	--[d]	--
	10^5	116	15	--	--
	10^6	267	17	3	15
	10^7	202	17	3	24
	10^8	240	29	12	31
VC-2	10^4	64	7	--	--
	10^5	86	8	--	--
	10^6	136	17	--	--
	10^7	167	21	6	10
	10^8	155	32	6	8
NYVAC	10^4	--	--	--	--
	10^5	--	--	--	--
	10^6	--	--	--	--
	10^7	--	--	--	--
	10^8	--	--	--	--

a   pfu of indicated vaccinia virus in 0.1 ml PBS
     inoculated intradermally into one site.

b   mean maximum size of lesions (mm^2)

c   mean time after inoculation for complete healing
     of lesion.

d   no lesions discernable.

Table 55.   Persistence of poxviruses at the site of intradermal inoculation

Virus	Inoculum Dose	Total Virus Recovered
WR	8.0^a	6.14
	7.0	6.26
	6.0	6.21
WYETH	8.0	3.66
	7.0	4.10
	6.0	3.59
VC-2	8.0	4.47
	7.0	4.74
	6.0	3.97
NYVAC	8.0	0
	7.0	0
	6.0	0

a: expressed as $\log_{10}$ pfu.

Table 56.   Virulence studies in immunocompromised mice

Poxvirus Strain	LD_{50}[a]	
	Nude mice	Cyclophosphamide treated mice
WR	422	42
VC-2	$>10^9$	$<1.65 \times 10^5$
WYETH	1.58×10^7	1.83×10^6
NYVAC	$>5.50 \times 10^8$	7.23×10^8
ALVAC	$>10^9$	$\geq 5.00 \times 10^{8b}$

a: Calculated 50% lethal dose (pfu) for nude or
     cyclophosphamide treated mice by the indicated vaccinia
     viruses and for ALVAC by intraperitoneal route.

b: 5 out of 10 mice died at the highest dose of 5×10^8
     pfu.

Table 57.   Comparative efficacy of NYVAC-RG and ALVAC-RG
             in mice

Recombinant	PD_{50}[a]
VV-RG	3.74
ALVAC-RG	3.86
NYVAC-RG	3.70

a: Four to six week old mice were inoculated in the
     footpad with 50-100μl of a range of dilutions (2.0 -
     8.0 $\log_{10}$ tissue culture infection dose 50% ($TCID_{50}$) of
     either the VV-RG (Kieny et al., 1984), ALVAC-RG (vCP65)
     or NYVAC-RG (vP879). At day 14, mice of each group
     were challenged by intracranial inoculation of 30μl of
     a live CVS strain rabies virus corresponding to 15
     lethal dose 50% (LD_{50}) per mouse. At day 28, surviving
     mice were counted and a protective dose 50% (PD_{50}) was
     calculated.

Having thus described in detail preferred embodiments
of the present invention, it is to be understood that the
invention defined by the appended claims is not to be
limited to particular details set forth in the above
description as many apparent variations thereof are possible
without departing from the spirit or scope of the present
invention.

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WHAT IS CLAIMED IS:

      1.   A modified recombinant virus, said modified recombinant virus having virus-encoded genetic functions associated with virulence inactivated therein so that the virus has attenuated virulence.

      2.   A virus as claimed in claim 1, wherein said virus is a poxvirus.

      3.   A virus as claimed in claim 2, further comprising exogenous DNA from a non-poxvirus source in a nonessential region of the poxvirus genome.

      4.   A virus as claimed in claim 3, wherein said poxvirus is vaccinia virus and the exogenous DNA is from a non-vaccinia source.

      5.   A virus as claimed in claim 2, wherein the poxvirus is vaccinia virus and the genetic functions are inactivated by deleting an open reading frame encoding a virulence factor.

      6.   A virus as claimed in claim 2, wherein the poxvirus is vaccinia virus and the genetic functions are inactivated by insertional inactivation of an open reading frame encoding a virulence factor.

      7.   A virus as claimed in claim 5, wherein the open reading frame is selected from the group consisting of J2R, B13R + B14R, A26L, A56R, C7L - K1L, and I4L and combinations thereof.

      8.   A virus as claimed in claim 6, wherein the open reading frame is selected from the group consisting of J2R, B13R + B14R, A26L, A56R, C7L - K1L, and I4L and combinations thereof.

      9.   A virus as claimed in claim 5, wherein the open reading frame comprises a thymidine kinase gene, a hemorrhagic region, an A type inclusion body region, a hemagglutinin gene, a host range gene region or a large subunit, ribonucleotide reductase.

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10.   A virus as claimed in claim 6, wherein the
open reading frame comprises a thymidine kinase gene, a
hemorrhagic region, an A type inclusion body region, a
hemagglutinin gene, a host range gene region or a large
subunit, ribonucleotide reductase.

11.   A virus as claimed in claim 1, which is
vP410, vP553, vP618, vP723, vP804 or vP866.

12.   A virus as claimed in claim 1, which is
vP796, vP938, vP953, vP977 or vP954.

13.   A virus as claimed in claim 1, which is
NYVAC.

14.   A recombinant vaccinia virus as claimed in
claim 4 wherein the non-vaccinia source is selected from the
group consisting of rabies virus, Hepatitis B virus,
Japanese encephalitis virus, yellow fever virus, Dengue
virus, measles virus, pseudorabies virus, Epstein-Barr
virus, herpes simplex virus, human immunodeficiency virus,
simian immunodeficiency virus, equine herpes virus, bovine
herpes virus, bovine viral diarrhea virus, human
cytomegalovirus, canine parvovirus, equine influenza virus,
feline leukemia virus, feline herpes virus, Hantaan virus,
C. tetani, avian influenza virus, mumps virus and Newcastle
Disease virus.

15.   A recombinant vaccinia virus as claimed in
claim 14, wherein the non-vaccinia source is rabies virus
and the recombinant vaccinia virus is vP879 or vP999.

16.   A recombinant vaccinia virus as claimed in
claim 14, wherein the non-vaccinia source is Hepatitis B
virus and the recombinant vaccinia virus is vP856, vP896,
vP897, vP858, vP891, vP932, vP975, vP930, vP919, vP941 or
vP944.

17.   A recombinant vaccinia virus as claimed in
claim 14, wherein the non-vaccinia source is Japanese
encephalitis virus and the recombinant vaccinia virus is
vP555, vP825, vP908, vP923, vP857, vP864 or vP829.

18.   A recombinant vaccinia virus as claimed in
claim 14, wherein the non-vaccinia source is yellow fever

virus and the recombinant vaccinia virus is vP766, vP764, vP869, vP729, vP725, vP997, or vP984.

20. 19. A recombinant vaccinia virus as claimed in claim 14, wherein the non-vaccinia source is Dengue virus and the recombinant vaccinia virus is vP867, vP962 or vP955.

20. A recombinant vaccinia virus as claimed in claim 14, wherein the non-vaccinia source is measles virus and the recombinant vaccinia virus is vP913 or vP997.

21. A recombinant vaccinia virus as claimed in claim 14, wherein the non-vaccinia source is pseudorabies virus and the recombinant vaccinia virus is vP881, vP883, vP900, vP912, vP925, vP915 or vP916.

22. A recombinant vaccinia virus as claimed in claim 14, wherein the non-vaccinia source is Epstein-Barr virus and the recombinant vaccinia virus is vP941 or vP944.

23. A recombinant vaccinia virus as claimed in claim 14, wherein the non-vaccinia source is herpes simplex virus and the recombinant vaccinia virus is vP914.

24. A recombinant vaccinia virus as claimed in claim 14, wherein the non-vaccinia source is human immunodeficiency virus and the recombinant vaccinia virus is vP911, vP921, vP878, vP939, vP940, vP920, vP922, vP1008, vP1004, vP1020, vP1078, vP994, vP1036, vP1035, vP969, vP989, vP991, vP990, vP970, vP973, vP971, vP979, vP978, vP988, vP1009, vP1062, vP1061, vP1060, vP1084, vP1045, vP1047 or vP1044.

25. A recombinant vaccinia virus as claimed in claim 14, wherein the non-vaccinia source is simian immunodeficiency virus and the recombinant vaccinia virus is vP873, vP948, vP943, vP942, vP952, vP948, vP1042, vP1071, vP943, vP942, vP952 or vP1050.

26. A recombinant vaccinia virus as claimed in claim 14 wherein the non-vaccinia source is equine herpes virus and the recombinant vaccinia virus is vP1043, vP1025 or vP956.

27. A recombinant vaccinia virus as claimed in claim 14 wherein the non-vaccinia source is bovine herpes

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virus and the recombinant vaccinia virus is vP1051, vP1074, vP1073, vP1083, vP1087 or vP1079.

28. A recombinant vaccinia virus as claimed in claim 14 wherein the non-vaccinia source is bovine viral diarrhea virus and the recombinant vaccinia virus is vP972, vP1017 or vP1097.

29. A recombinant vaccinia virus as claimed in claim 14 wherein the non-vaccinia source is human cytomegalovirus and the recombinant vaccinia virus is vP1001.

30. A recombinant vaccinia virus as claimed in claim 14 wherein the non-vaccinia source is canine parvovirus and the recombinant vaccinia virus is vP998 or vP999.

31. A recombinant vaccinia virus as claimed in claim 14 wherein the non-vaccinia source is equine influenza virus and the recombinant vaccinia virus is vP961 or vP1063.

32. A recombinant vaccinia virus as claimed in claim 14 wherein the non-vaccinia source is feline leukemia virus and the recombinant vaccinia virus is vP1011.

33. A recombinant vaccinia virus as claimed in claim 14 wherein the non-vaccinia source is Hantaan virus and the recombinant vaccinia virus is vp882, vP950 or vP951.

34. A recombinant vaccinia virus as claimed in claim 14 wherein the non-vaccinia source is *C. tetani* and the recombinant vaccinia virus is vp1075.

35. A poxvirus having virus-encoded genetic functions inactivated therein so that the virus has attenuated virulence; said poxvirus further comprising exogenous DNA from a non-poxvirus source inserted by recombination in a nonessential region of the poxvirus genome.

36. A poxvirus as claimed in claim 35 wherein the non-poxvirus source is selected from the group consisting of rabies virus, Hepatitis B virus, Japanese encephalitis virus, yellow fever virus, Dengue virus, measles virus, pseudorabies virus, Epstein-Barr virus, herpes simplex

virus, human immunodeficiency virus, simian immunodeficiency
virus, equine herpes virus, bovine herpes virus, bovine
viral diarrhea virus, human cytomegalovirus, canine
parvovirus, equine influenza virus, feline leukemia virus,
feline herpes virus, Hantaan virus, *C. tetani*, avian
influenza virus, mumps virus and Newcastle Disease virus.

37. A poxvirus as claimed in claim 36 wherein the
poxvirus is a canarypox virus, the non-poxvirus source is
rabies virus, and the canarypox virus is vCP65 or vCP136.

38. A poxvirus as claimed in claim 36 wherein the
poxvirus is a canarypox virus, the non-poxvirus source is
human immunodeficiency virus and the canarypox virus is
vCP95, vCP112, vCP60, vCP61, vCP125, vCP124, vCP126, vCP144,
vCP120, vCP138, vCP117, vCP130, vCP152, vCP155, vCP156,
vCP146, vCP148, vCP154, vCP168 or vCP153.

39. A poxvirus as claimed in claim 36 wherein the
poxvirus is a canarypox virus, the non-poxvirus source is
equine herpes virus and the canarypox virus is cP132.

40. A poxvirus as claimed in claim 36 wherein the
poxvirus is a canarypox virus, the non-poxvirus source is
human cytomegalovirus and the canarypox virus is vCP139.

41. A poxvirus as claimed in claim 36 wherein the
poxvirus is a canarypox virus, the non-poxvirus source is
canine parvovirus and the canarypox virus is vCP123 or
vCP136.

42. A poxvirus as claimed in claim 36 wherein the
poxvirus is a canarypox virus, the non-poxvirus source is
Epstein-Barr virus and the canarypox virus is vCP167.

43. A poxvirus as claimed in claim 36 wherein the
poxvirus is a canarypox virus, the non-poxvirus source is
equine influenza virus and the canarypox virus is vCP128 or
vCP159.

44. A poxvirus as claimed in claim 36 wherein the
poxvirus is a canarypox virus, the non-poxvirus source is
feline leukemia virus and the canarypox is vCP177, vCP83,
vCP35, vCP37, vCP87, vCP93 or vCP97.

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45. A poxvirus as claimed in claim 36 wherein the poxvirus is a canarypox virus, the non-poxvirus source is feline herpes virus and the canarypox virus is vCP162.

46. A poxvirus as claimed in claim 36 wherein the poxvirus is a canarypox virus, the non-poxvirus source is Hantaan virus and the canary pox virus is vCP114 or vCP119.

47. A poxvirus as claimed in claim 36 wherein the poxvirus is a canarypox virus, the non-poxvirus source is Hepatitis B virus and the canarypox virus is vCP169 or vCP157.

48. A poxvirus as claimed in claim 36 wherein the poxvirus is a canarypox virus, the non-poxvirus source is *C. tetani* and the canarypox virus is vCP161.

49. A poxvirus as claimed in claim 36 wherein the poxvirus is a canarypox virus, the non-poxvirus source is mumps virus and the canarypox virus is vCP171.

50. A poxvirus as claimed in claim 36 wherein the poxvirus is a canarypox virus, the non-poxvirus source is Japanese encephalitis virus, and the canarypox virus is vCP107 or vCP140.

51. A poxvirus as claimed in claim 36 wherein the poxvirus is a canarypox virus, the non-poxvirus source is simian immunodeficiency virus, and the canarypox virus is vCP172.

52. A poxvirus as claimed in claim 36 wherein the poxvirus is a fowlpox virus, the non-poxvirus source is avian influenza virus and the fowlpox virus is vFP89, vFP92, vFP100 or vFP122.

53. A poxvirus as claimed in claim 36 wherein the poxvirus is a fowlpox virus, the non-poxvirus source is human immunodeficiency virus and the fowlpox virus is vFP62, vFP63 or vFP174.

54. A poxvirus as claimed in claim 36 wherein the poxvirus is a fowlpox virus, the non-poxvirus is a fowlpox virus, the non-poxvirus source is Newcastle Disease virus and the fowlpox virus is vFP96.

55. A rabies virus recombinant canarypox virus which is vCP65.

56. A human immunodeficiency virus recombinant canarypox virus which is vCP95, vCP112, vCP60 or vCP61.

57. A human immunodeficiency virus recombinant fowlpox virus which is vFP62 or vFP63.

58. An avian influenza virus recombinant fowlpox virus which is vFP89, vFP92, vFP100 or vFP122.

59. A vaccine for inducing an immunological response in a host animal inoculated with the vaccine, said vaccine comprising a carrier and a recombinant virus as claimed in any one of claims 4, 35, 55, 56 and 57.

60. A vaccine for inducing an immunological response in a human inoculated with the vaccine, said vaccine comprising a carrier and a recombinant virus as claimed in any one of claims 4, 35 or 55.

61. A method for expressing a gene product in a cell cultured *in vitro*, which method comprises introducing into the cell a modified recombinant virus as claimed in claim 4.

62. A modified vector for expressing a gene product in a host, wherein the vector is modified so that it has attenuated virulence in the host, said modified vector comprising DNA which codes for and expresses the gene product in the host.

63. A vector as claimed in claim 62, wherein said vector is a poxvirus.

64. A vector as claimed in claim 63, wherein said poxvirus is vaccinia virus, canarypox virus or fowlpox virus.

65. A vaccine for inducing an immunological response in a host animal inoculated with said vaccine, said vaccine comprising a carrier and a modified vector as claimed in claim 62.

66. A method for expressing a gene product in a cell cultured *in vitro*, which method comprises introducing into the cell a modified vector as claimed in claim 62.

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FIG. I

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 6

FIG. 7
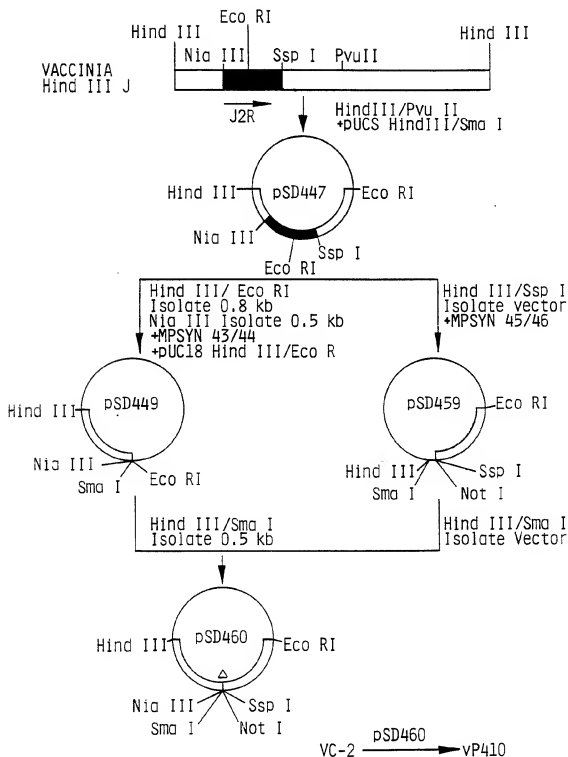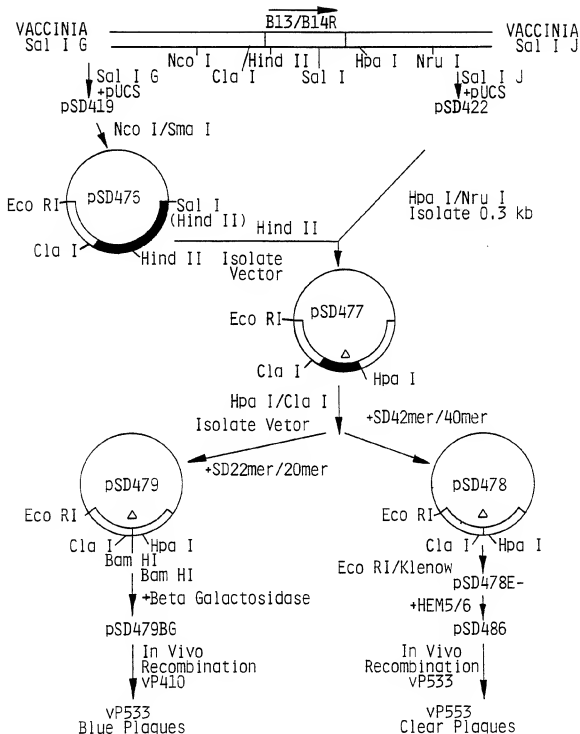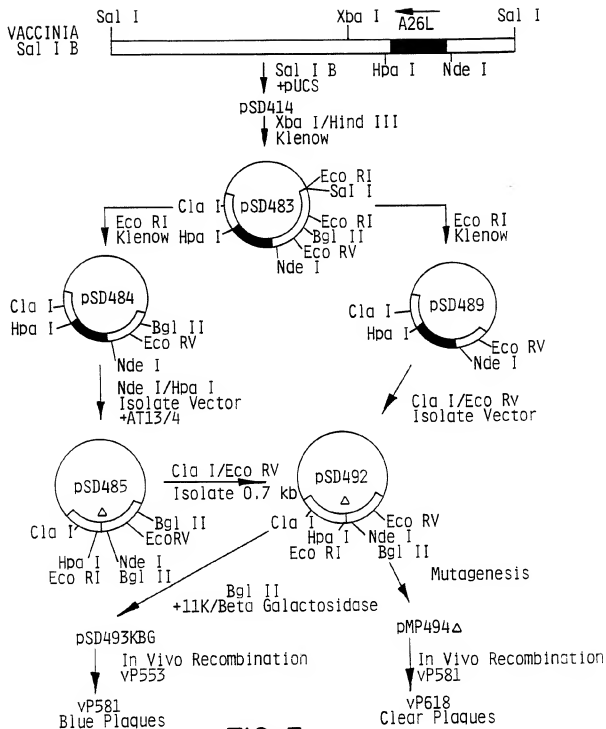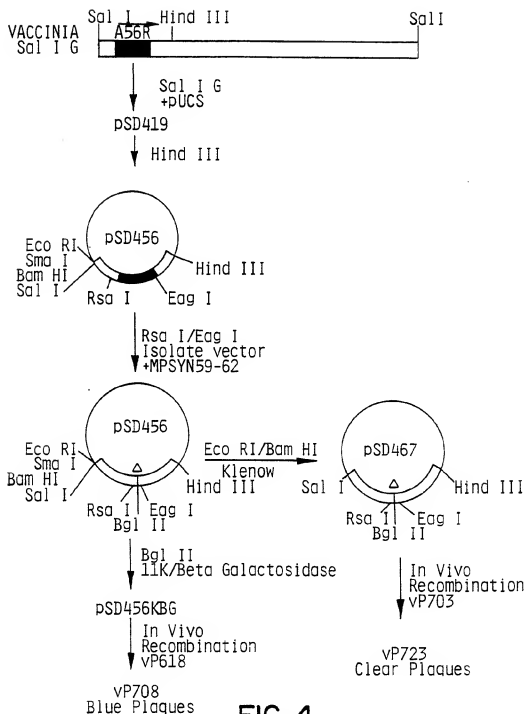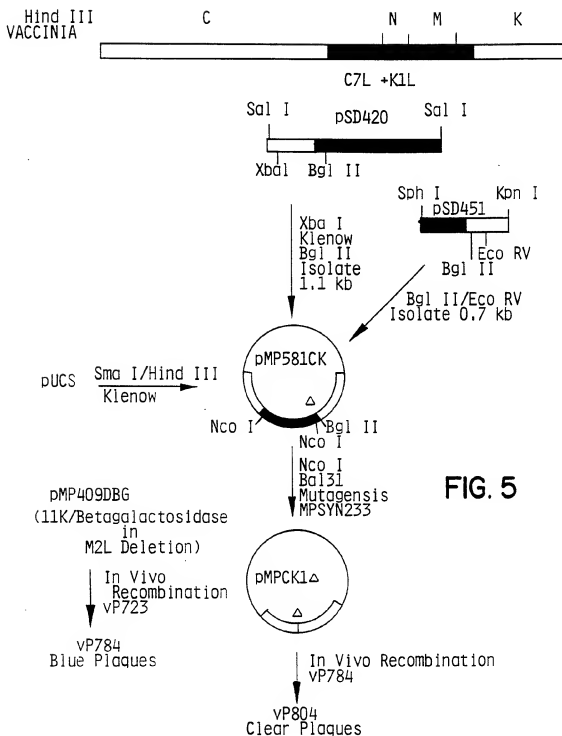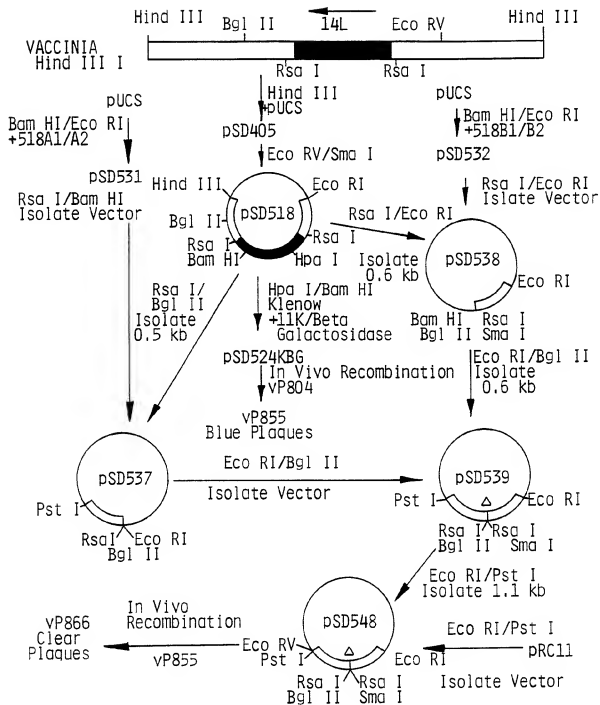
FIG. 8

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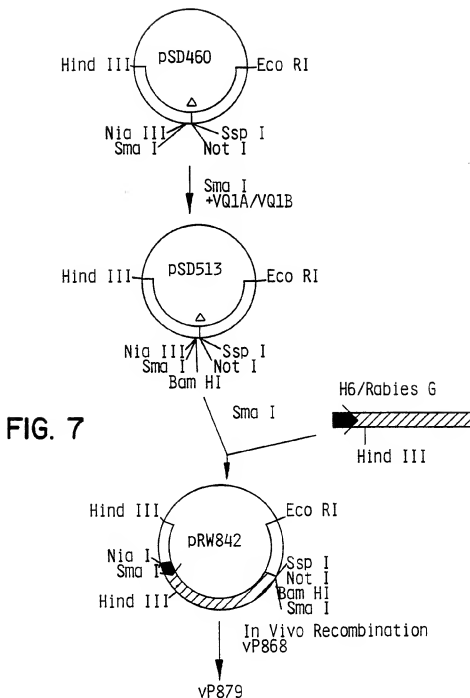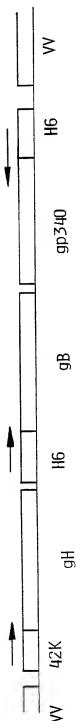
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         10        20        30        40        50        60
TTCTTTATTCTATACTTAAAAAGTGAAAATAAATACAAAGGTTCTTGAGGGTTGTGTTAA

         70        80        90       100       110       120
ATTGAAAGCGAGAAATAATCATAAATTATTTCATTATCGCGATATCCGTTAAGTTTGTAT

        130       140       150       160       170       180
GCTAATGCAGTGGAATTCTACTACTTTTCACCAAACTTTACAAGATCCTAGAGTAAGAGG
     M  Q  W  N  S  T  T  F  H  Q  T  L  Q  D  P  R  V  R  G
     S2

        190       200       210       220       230       240
ATTATATTTTCCTGCTGGAGGATCTTCTTCTGGAGCTGTAAATCCTGTACCTACTACTGC
 L  Y  F  P  A  G  G  S  S  S  G  A  V  N  P  V  P  T  T  A>

        250       260       270       280       290       300
TTCTCCTTTATCTTCTATTTTTAGTAGAATTGGAGATCCTGCTTTAAATATGGAAAATAT
 S  P  L  S  S  I  F  S  R  I  G  D  P  A  L  N  M  E  N  I
                                                    S

        310       320       330       340       350       360
TACTTCTGGATTTTTAGGACCTTTATTAGTATTACAAGCTGGATTCTTTCTATTAACTAG
 T  S  G  F  L  G  P  L  L  V  L  Q  A  G  F  F  L  L  T  R>

        370       380       390       400       410       420
AATTTTAACTATTCCTCAATCTCTAGATTCTTGGTGGACTTCTTTAAATTTTTTAGGAGG
 I  L  T  I  P  Q  S  L  D  S  W  W  T  S  L  N  F  L  G  G

        430       440       450       460       470       480
AACTACTGTTTGTTTAGGACAAAATTCTCAATCTCCTACTTCTAATCACTCTCCTACTTC
 T  T  V  C  L  G  Q  N  S  Q  S  P  T  S  N  H  S  P  T  S>
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FIG. 9A

```
         490       500       510       520       530       540
TTGTCCTCCTACTTGTCCTGGATATCGTTGGATGTGTTTAAGAAGATTTATTATTTTCTT
  C  P  P  T  C  P  G  Y  R  W  M  C  L  R  R  F  I  I  F  L

         550       560       570       580       590       600
ATTTATTTTATTATTGTGTTTAATTTTCTTATTAGTATTATTAGATTATCAAGGAATGTT
  F  I  L  L  L  C  L  I  F  L  L  V  L  L  D  Y  Q  G  M  L>

         610       620       630       640       650       660
ACCTGTTTGTCCTTTAATTCCTGGATCCTCTACTACTTCTACTGGACCTTGTAGAACTTG
  P  V  C  P  L  I  P  G  S  S  T  T  S  T  G  P  C  R  T  C

         670       680       690       700       710       720
TATGACTACTGCTCAAGGAACTTCTATGTATCCTTCTTGTTGTGTACTAAACCTTCTGA
  M  T  T  A  Q  G  T  S  M  Y  P  S  C  C  C  T  K  P  S  D>

         730       740       750       760       770       780
CGGAAATTGTACTTGTATTCCTATTCCTTCTTCTTGGGCTTTTGGAAAATTCTTGTGGGA
  G  N  C  T  C  I  P  I  P  S  S  W  A  F  G  K  F  L  W  E

         790       800       810       820       830       840
GTGGGCTTCTGCTAGATTTTCTTGGTTATCTTTATTAGTACCTTTTGTACAGTGGTTTGT
  W  A  S  A  R  F  S  W  L  S  L  L  V  P  F  V  Q  W  F  V>

         850       860       870       880       890       900
AGGATTATCTCCTACTGTTTGGTTATCTGTAATTTGGATGATGTGGTATTGGGGACCTTC
  G  L  S  P  T  V  W  L  S  V  I  W  M  M  W  Y  W  G  P  S

         910       920       930       940       950       960
TTTATATTCTATTTTATCTCCTTTTTTACCTTTATTACCTATTTTCTTTTGTTTGTGGGT
  L  Y  S  I  L  S  P  F  L  P  L  L  P  I  F  F  C  L  W  V>

         970
ATATATTTAA
  Y  I  *
```

FIG. 9B

FIG. IOA

FIG. IOB
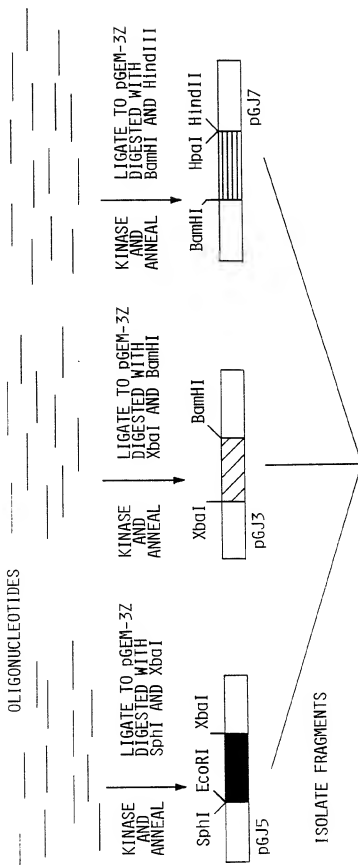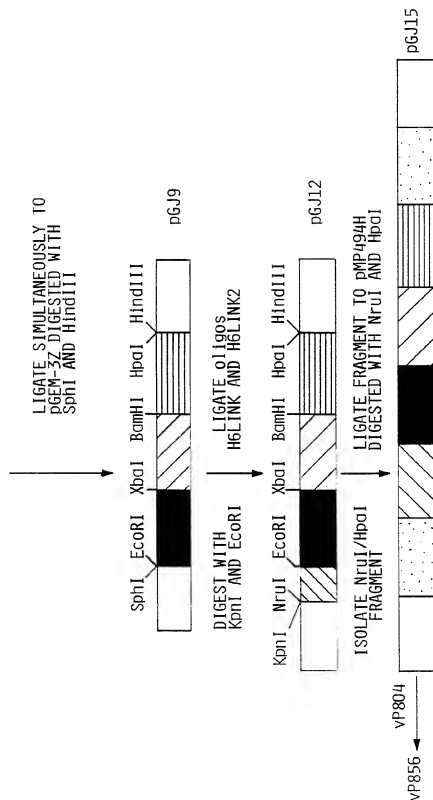
```
        10        20        30        40        50        60
GTAGACTGTTTATACAAGATTGAAAATATATTTCTTTTTATTGAGTGGTGGTAGTTACGG

        70        80        90       100       110       120
ATATCTAATATTAATATTAGACTATCTCTATCGTCACACAACAAAATCGATTGCCATGGG
                                                          M  G>
                                                          S1

       130       140       150       160       170       180
GCAGAATCTTTCCACCAGCAATCCTCTGGGATTCTTTCCCGACCACCAGTTGGATCCAGC
  Q  N  L  S  T  S  N  P  L  G  F  F  P  D  H  Q  L  D  P  A

       190       200       210       220       230       240
CTTCAGAGCAAACAACGCAAATCCAGATTGGGACTTCAATCCCAACAAGGACACCTGGCC
  F  R  A  N  N  A  N  P  D  W  D  F  N  P  N  K  D  T  W  P>

       250       260       270       280       290       300
AGACGCCAACAAGGTAGGAGCTGGAGCATTCGGGCTGGGTTTCACCCCACCGCACGGAGG
  D  A  N  K  V  G  A  G  A  F  G  L  G  F  T  P  P  H  G  G

       310       320       330       340       350       360
CCTTTTGGGGTGGAGCCCTCAGGCTCAGGGCATACTACAAATTTGCCAGCAAATCCGCC
  L  L  G  W  S  P  Q  A  Q  G  I  L  Q  T  L  P  A  N  P  P>

       370       380       390       400       410       420
TCCTGCCTCCACCAATCGCCAGACAGGAAGGCAGCCTACCCCGCTGTCTCCACCTTTGAG
  P  A  S  T  N  R  Q  T  G  R  Q  P  T  P  L  S  P  P  L  R

       430       440       450       460       470       480
AAACACTCATCCTCCAGGCCATGCAGTGGAATTCCACAACCTTTCACCAAACTCTGCAAGA
  N  T  H  P  Q  A  M  Q  W  N  S  T  T  F  H  Q  T  L  Q  D>
                        S2

       490       500       510       520       530       540
TCCCCAGAGTGAGAGGCCTGTATTTCCCTGCTGGTGGCTCCAGTTCAGGAGCAGTAAACCC
  P  R  V  R  G  L  Y  F  P  A  G  G  S  S  S  G  A  V  N  P

       550       560       570       580       590       600
TGTTCCGACTACTGCCTCTCCCTTATCGTCAATCTTCTCGAGGATTGGGGACCCTGCGCT
  V  P  T  T  A  S  P  L  S  S  I  F  S  R  I  G  D  P  A  L>

       610       620       630       640       650       660
GAACATGGAGAACATCACATCAGGATTCCTAGGACCCCTTCTCGTGTTACAGGCGGGGTT
  N  M  E  N  I  T  S  G  F  L  G  P  L  L  V  L  Q  A  G  F
     S
```

FIG. IIA

```
      670       680       690       700       710       720
CTTCTTGTTGACAAGAATCCTCACAATACCGCAGAGTCTAGACTCGTGGTGGACTTCTCT
 F  L  L  T  R  I  L  T  I  P  Q  S  L  D  S  W  W  T  S  L >

      730       740       750       760       770       780
CAATTTTCTAGGGGGAACTACCGTGTGTCTTGGCCAAAATTCGCAGTCCCCAACCTCCAA
 N  F  L  G  G  T  T  V  C  L  G  Q  N  S  Q  S  P  T  S  N

      790       800       810       820       830       840
TCACTCACCAACCTCCTGTCCTCCAACTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCG
 H  S  P  T  S  C  P  P  T  C  P  G  Y  R  W  M  C  L  R  R >

      850       860       870       880       890       900
TTTTATCATCTTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGGA
 F  I  I  F  L  F  I  L  L  L  C  L  I  F  L  L  V  L  L  D

      910       920       930       940       950       960
CTATCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCTCAACCACCAGCACGGG
 Y  Q  G  M  L  P  V  C  P  L  I  P  G  S  S  T  T  S  T  G >

      970       980       990      1000      1010      1020
ACCATGCCGAACCTGCATGACTACTGCTCAAGGAACCTCTATGTATCCCTCCTGTTGCTG
 P  C  R  T  C  M  T  T  A  Q  G  T  S  M  Y  P  S  C  C  C

     1030      1040      1050      1060      1070      1080
TACCAAACCTTCGGACGGAAATTGCACCTGTATTCCCATCCCATCATCCTGGGCTTTCGG
 T  K  P  S  D  G  N  C  T  C  I  P  I  P  S  S  W  A  F  G >

     1090      1100      1110      1120      1130      1140
AAAATTCCTATGGGAGTGGGCCTCAGCCCGTTTCTCCTGGCTCAGTTTACTAGTGCCATT
 K  F  L  W  E  W  A  S  A  R  F  S  W  L  S  L  L  V  P  F

     1150      1160      1170      1180      1190      1200
TGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTTATATGGATGATGTG
 V  Q  W  F  V  G  L  S  P  T  V  W  L  S  V  I  W  M  M  W >

     1210      1220      1230      1240      1250      1260
GTATTGGGGGCCAAGTCTGTACAGCATCTTGAGTCCCTTTTTACCGCTGTTACCAATTTT
 Y  W  G  P  S  L  Y  S  I  L  S  P  F  L  P  L  L  P  I  F

     1270      1280
CTTTTGTCTTTGGGTATACATTTAA >
 F  C  L  W  V  Y  I  *  >
```

FIG. IIB

FIG. I2A
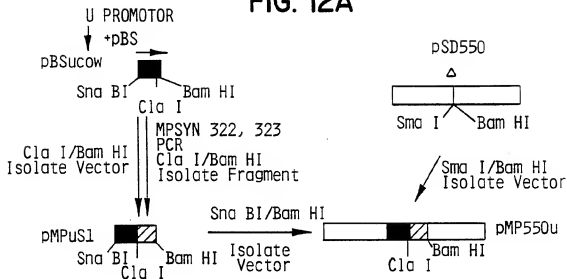
FIG. I2B
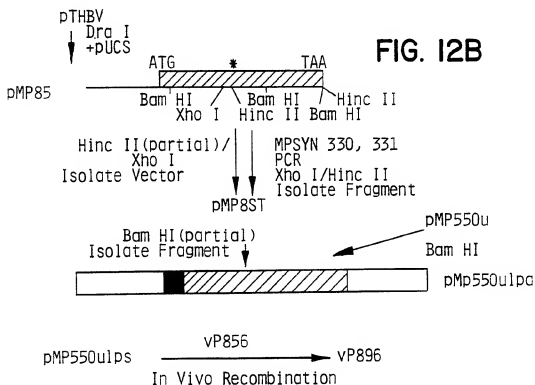
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```
         10        20        30        40        50        60
TACATCATGCAGTGGTTAAACAAAAACATTTTTATTCTCAAATGAGATAAAGTGAAAATA

         70        80        90       100       110       120
TATATCATTATATTACAAAGTACAATTATTTAGGTTTAATCATGGGAACGAACCTATCTG
                                              M  G  T  N  L  S
                                              S1

        130       140       150       160       170       180
TTCCCAACCCACTTGGATTTTTTCCTGATCATCAGTTAGACCCTGCTTTCGGAGCCAACT
 V  P  N  P  L  G  F  F  P  D  H  Q  L  D  P  A  F  G  A  N

        190       200       210       220       230       240
CAAACAATCCTGACTGGGATTTTAACCCCGTCAAAGACGATTGGCCTGCAGCCAACCAAG
 S  N  N  P  D  W  D  F  N  P  V  K  D  D  W  P  A  A  N  Q

        250       260       270       280       290       300
TAGGTGTGGGAGCTTTCGGACCAAGGCTCACTCCTCCACACGGCGGTATATTAGGTTGGT
 V  G  V  G  A  F  G  P  R  L  T  P  P  H  G  G  I  L  G  W

        310       320       330       340       350       360
CTCCACAAGCTCAAGGCATATTGACCACAGTGTCAACAATTCCTCCACCAGCCTCTACTA
 S  P  Q  A  Q  G  I  L  T  T  V  S  T  I  P  P  P  A  S  T

        370       380       390       400       410       420
ATCGGCAGTCTGGTAGACAGCCAACTCCCATCTCTCCTCCTCTAAGAGACAGTCACCCAC
 N  R  Q  S  G  R  Q  P  T  P  I  S  P  P  L  R  D  S  H  P

        430       440       450       460       470       480
AAGCTATGCAGTGGAATTCAACTGCTTTTCACCAGCACTTCAAGACCCTAGAGTCAGGG
 Q  A  M  Q  W  N  S  T  A  F  H  Q  T  L  Q  D  P  R  V  R
      S2

        490       500       510       520       530       540
GTCTATATCTTCCTGCAGGTGGATCTAGTTCTGGAACTGTAAACCCAGCTCCGAATATTG
 G  L  Y  L  P  A  G  G  S  S  S  G  T  V  N  P  A  P  N  I

        550       560       570       580       590       600
CCAGTCACATCTCGTCTATCTCCGCGAGGACTGGAGACCCAGTGACGAACATGGACATCG
 A  S  H  I  S  S  I  S  A  R  T  G  D  P  V  T  N  M  D  I
                                                     core
```

FIG. 13A

```
          610        620        630        640        650        660
ACCCTTATAAAGAATTTGGAGCTACTGTGGAGTTACTCTCGTTTTTGCCTTCTGACTTCT
D   P   Y   K   E   F   G   A   T   V   E   L   L   S   F   L   P   D   S   F

          670        680        690        700        710        720
TTCCTTCAGTACGAGATCTTCTAGATACCGCCTCAGCTCTGTATCGGGGAAGCCTTAGAGT
F   P   S   V   R   D   L   L   D   T   A   S   A   L   Y   R   E   A   L   E

          730        740        750        760        770        780
CTCCTGAGCATTGTTCACCTCACCATACTGCACTCAGGCAAGCAATTCTTTGCTGGGGGG
S   P   E   H   C   S   P   H   H   T   A   L   R   Q   A   I   L   C   W   G

          790        800        810        820        830        840
AACTAATGACTCTAGCTACCTGGGTGGGTGTTAATTTGGAAGATCCAGCATCTAGAGACC
E   L   M   T   L   A   T   W   V   G   V   N   L   E   D   P   A   S   R   D

          850        860        870        880        890        900
TAGTAGTCAGTTATGTCAACACTAATATGGGCCTAAAGTTCAGGCAACTCTTGTGGTTTC
L   V   V   S   Y   V   N   T   N   M   G   L   K   F   R   Q   L   L   W   F

          910        920        930        940        950        960
ACATTTCTTGTCTCACTTTTGGAAGAGAAACCGTTATAGAGTATTTGGTGTCTTTCGGAG
H   I   S   C   L   T   F   G   R   E   T   V   I   E   Y   L   V   S   F   G

          970        980        990       1000       1010       1020
TGTGGATTCGCACTCCTCCAGCTTATAGACCACCAAATGCCCCTATCCTATCAACACTTC
V   W   I   R   T   P   P   A   Y   R   P   P   N   A   P   I   L   S   T   L

         1030       1040       1050       1060       1070       1080
CGGAAACTACTGTTGTTAGACGACGAGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTC
P   E   T   T   V   V   R   R   R   G   R   S   P   R   R   R   T   P   S   P

         1090       1100       1110       1120       1130       1140
GCAGACGAAGGTCTCAATCGCCGCGTCGCAGAAGATCTCAATCTCGGGAATCTCAATGTTAG
R   R   R   R   S   Q   S   P   R   R   R   R   S   Q   S   R   E   S   Q   C   *
```

FIG. 13B
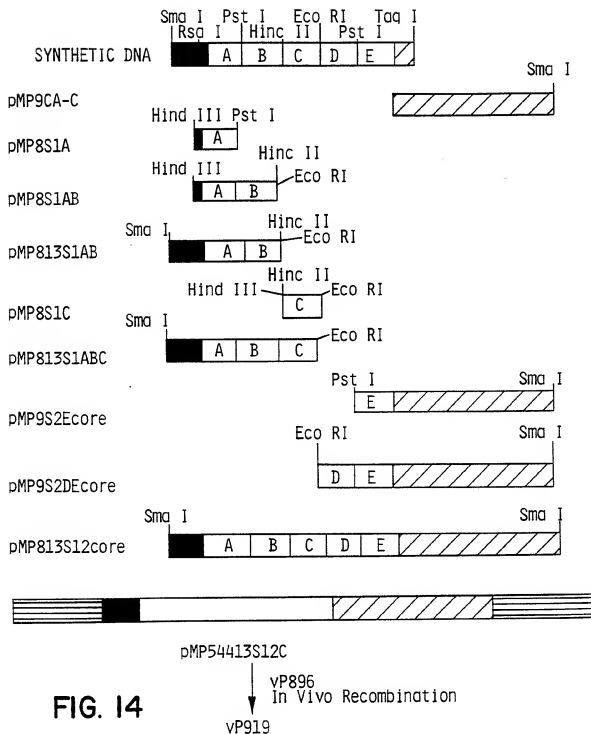
FIG. 14

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```
          10        20        30        40        50        60
CAAAATTGAAAATATATAATTACAATATAAAATGGGGCAGAATCTTTCCACCAGCAATCC
                                M  G  Q  N  L  S  T  S  N  P
                                S1

          70        80        90       100       110       120
TCTGGGATTCTTTCCCGACCACCAGTTGGATCCAGCCTTCAGAGCAAACAACGCAAATCC
 L  G  F  F  P  D  N  Q  L  D  P  A  F  R  A  N  N  A  N  P>

         130       140       150       160       170       180
AGATTGGGACTTCAATCCCAACAAGGACACCTGGCCAGACGCCAACAAGGTAGGAGCTGG
 D  W  D  F  N  P  N  K  D  T  W  P  D  A  N  K  V  G  A  G

         190       200       210       220       230       240
AGCATTCGGGCTGGGTTTCACCCCACCGCACGGAGGCCTTTTGGGGTGGAGCCCTCAGGC
 A  F  G  L  G  F  T  P  P  N  G  G  L  L  G  W  S  P  Q  A>

         250       260       270       280       290       300
TCAGGGCATACTACAAACTTTGCCAGCAAATCCGCCTCCTGCCTCCACCAATCGCCAGAC
 Q  S  I  L  Q  T  L  P  A  N  P  P  P  A  S  T  N  R  Q  T

         310       320       330       340       350       360
AGGAAGGCAGCCTACCCGCTGTCTCCACCTTTGAGAAACACTCATCCTCAGGCCATGCA
 G  R  Q  P  T  P  L  S  P  P  L  R  N  T  H  P  Q  A  M  Q>
                                                        S2

         370       380       390       400       410       420
GTGGAATTCCACAACCTTTCACCAAACTCTGCAAGATCCCAGAGTGAGAGGCCTGTATTT
 W  N  S  T  T  F  N  Q  T  L  Q  D  P  R  V  R  G  L  Y  F

         430       440       450       460       470       480
CCCTGCTGGTGGCTCCAGTTCAGGAGCAGTAAACCCTGTTCCGACTACTGCCTCTCCCTT
 P  A  G  G  S  S  S  G  A  V  N  P  V  P  T  T  A  S  P  L>

         490       500       510       520       530       540
ATCGTCAATCTTCTCGAGGATTGGGGACCCTGCGCTGAACATGGAGAACATCACATCAGG
 S  S  I  F  S  R  I  G  D  P  A  L  N  M  E  N  I  T  S  G
                                             S

         550       560       570       580       590       600
ATTCCTAGGACCCCTTCTCGTGTTACAGGCGGGGTTCTTCTTGTTGACAAGAATCCTCAC
 F  L  G  P  L  L  V  L  Q  A  G  F  F  L  L  T  R  I  L  T>

         610       620       630       640       650       660
AATACCGCAGAGTCTAGACTCGTGGTGGGACTTCTCTCAATTTTCTAGGGGGAACTACCGT
 I  P  Q  S  L  D  S  W  W  T  S  L  N  F  L  G  G  T  T  V

         670       680       690       700       710       720
GTGTCTTGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCC
 C  L  G  Q  N  S  Q  S  P  T  S  N  H  S  P  T  S  C  P  P>
```

```
      730       740       750       760       770       780
AACTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATCTTCCTCTTCATCCTT
   T  C  P  G  Y  R  W  M  C  L  R  R  F  I  I  F  L  F  I  L


      790       800       810       820       830       840
GCTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTG
   L  L  C  L  I  F  L  L  V  L  L  D  Y  Q  G  M  L  P  V  C>


      850       860       870       880       890       900
TCCTCTAATTCCAGGATCCTCAACCACCAGCACGGGACCATGCCGAACCTGCATGACTAC
   P  L  I  P  G  S  S  T  T  S  T  G  P  C  R  T  C  M  T  T


      910       920       930       940       950       960
TGCTCAAGGAACCTCTATGTATCCCTCCTGTTGCTGTACCAAACCTTCGGACGGAAATTG
   A  Q  G  T  S  M  Y  P  S  C  C  C  T  K  P  S  D  G  N  C>


      970       980       990      1000      1010      1020
CACCTGTATTCCCATCCCATCATCCTGGGCTTTCGGAAAATTCCTATGGGAGTGGGCCTC
   T  C  I  P  I  P  S  S  W  A  F  G  K  F  L  W  E  W  A  S


     1030      1040      1050      1060      1070      1080
AGCCCGTTTCTCCTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTC
   A  R  F  S  W  L  S  L  L  V  P  F  V  Q  W  F  V  G  L  S>


     1090      1100      1110      1120      1130      1140
CCCCACTGTTTGGCTTTCAGTTATATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAG
   P  T  V  W  L  S  V  I  W  M  M  W  Y  W  G  P  S  L  Y  S


     1150      1160      1170      1180      1190      1200
CATCTTGAGTCCCTTTTTACCGCTGTTACCAATTTTCTTTTGTCTTTGGGTATACATTTAA
   I  L  S  P  F  L  P  L  L  P  I  F  F  C  L  W  V  Y  I  *>
```

FIG. I5B

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```
   1 TGAATGTTAA ATGTTATACT TTGGATGAAG CTATAAATAT GCATTGGAAA AATAATCCAT
  61 TTAAAGAAAG GATTCAAATA CTACAAAACC TAAGCGATAA TATGTTAACT AAGCTTATTC
 121 TTAACGACGC TTTAAATATA CACAAATAAA CATAATTTTT GTATAACCTA ACAAATAACT
 181 AAAACATAAA AATAATAAAA GGAAATGTAA TATCGTAATT ATTTTACTCA GGAATGGGGT
 241 TAAATATTTA TATCACGTGT ATATCTATAC TGTTATCGTA TACTCTTTAC AATTACTATT
 301 ACGAATATGC AAGGAGATAAT AAGATTACGT ATTTAAGAGA ATCTTGTCAT GATAATTGGG
 361 TACGACATAG TGATAAATGC TATTTCGCAT CGTTACATAA AGTCAGTTGG AAAGATGGAT
 421 TTGACAGATG TAACTTAATA GGTGCAAAAA TGTTAAATAA CAGCATTCTA TCGGAAGATA
 481 GGATACCAGT TATATTTATAC AAAAATCACT GGTTGGATAA AACAGATTCT GCAATATTCG
 541 TAAAAGATGA AGATTACTGC GAATTTGTAA ACTATGACAA TAAAAAGCCA TTTATCTCAA
 601 CGACATCGTG TAATTCTTCC ATGTTTTATG TATGTGTTTC AGATATTATG AGATTACTAT
 661 AAACTTTTTG TATACTTATA TTCCGTAAAC TATATTAATC ATGAAGAAAA TGAAAAAGTA
 721 TAGAAGCTGT TCACGAGCGG TTGTTGAAAA CAACAAAATT ATACATTCAA GATGGCTTAC
 781 ATATACGTCT GTGAGGCTAT CATGGATAAT GACAATGCAT CTCTAAATAG GTTTTTGGAC
 841 AATGGATTCG ACCCTAACAC GGAATATGGT ACTCTACAAT CTCCTCTTGA AATGGCTGTA
 901 ATGTTCAAGA ATACCGAGGC TATAAAAATC TTGATGAGGT ATGGAGCTAA ACCTGTAGTT
 961 ACTGAATGCA CAACTTCTTG TCTGCATGAT GCGGTGTTGA GAGACGACTA CAAAATAGTG
1021 AAAGATCTGT TGAAGAATAA CTATGTAAAC AATGTTCTTT ACAGCGGAGG CTTTACTCCT
1081 TTGTGTTTGG CAGCTTACCT TAACAAAGTT AATTTGGTTA AACTTCTATT GGCTCATTCG
1141 GCGGATGTAG ATATTTCAAA CACGGATCGG TTAACTCCTC TACATATAGC CGTATCAAAT
1201 AAAAATTTAA CAATGGTTAA ACTTCTATTG AACAAAGGTG CTGATACTGA CTTGCTGGAT
1261 AACATGGGAC GTACTCCTTT AATGATCGCT GTACAATCTG GAAATATTGA AATATGTAGC
1321 ACACTACTTA AAAAAAATAA AATGTCCAGA ACTGGGAAAA ATTGATCTTG CCAGCTGTAA
1381 TTCATGGTAG AAAAGAAGTG CTCAGGCTAC TTTCAACAA AGGAGCAGAT GTAAACTACA
1441 TCTTTGAAAG AAATGGAAAA TCATATACTG TTTTGGAATT GATTAAAGAA AGTTACTCTG
1501 AGACACAAAA GAGGTAGCTG AAGTGGTACT CTCAAAATGC AGAACGATGA CTGCGAAGCA
1561 AGAAGTAGAG AAATAACACT TTATGACTTT CTTAGTTGTA GAAAAGATAG AGATATAATG
1621 ATGGTCATAA ATAACTCTGA TATTGCAAGT AAATGCAATA ATAAGTTAGA TTTATTTAAA
1681 AGGATAGTTA AAAATAGAAA AAAAGAGTTA ATTTGTAGGG TTAAAATAAT ACATAAGATC
1741 TTAAAATTTA TAAATACGCA TAATAATAAA AATAGATTAT ACTTATTACC TTCAGAGATA
1801 AAATTTAAGA TATTTACTTA TTTAACTTAT AAAGATCTAA AATGCATAAT TTCTAAATAA
1861 TGAAAAAAAA GTACATCATG AGCAACGCGT TAGTATATTT TACAATGGAG ATTAACGCTC
1921 TATACCGTTC TATGTTTATT GATTCAGATG ATGTTTTAGA AAAGAAAGTT ATTGAATATG
1981 AAAACTTTAA TGAAGATGAA GATGACGACG ATGATTATTG TTGTAAATCT GTTTTAGATG
2041 AAGAAGATGA CGCGCTAAAG TATACTATGG TTACAAAGTA TAAGTCTATA CTACTAATGG
2101 CGACTTGTGC AAGAAGGTAT AGTATAGTGA AAATGTTGTT AGATTATGAT TATGAAAAAC
2161 CAAATAAATC AGATCCATAT CTAAAGGTAT CTCCTTTGCA CATAATTTCA TCTATTCCTA
2221 GTTTAGAATA CTTTTCATTA TATTTGTTTA CAGCTGAAGA CGAAAAAAAT ATATCGATAA
2281 TAGAAGATTA TGTTAACTCT GCTAATAAGA TGAAATTGAA TGAGTCTGTG ATAATAGCTA
2341 TAATCAGAGA AGTTCTAAAA GGAAATAAAA ATCTAACTGA TCAGGATATA AAAACATTGG
2401 CTGATGAAAT CAACAAGGAG GAACTGAATA TAGCTAAACT ATTGTTAGAT AGAGGGGCCA
2461 AAGTAAATTA CAAGGATGTT TACGGTTCTT CAGCTCTCCA TAGAGCTGCT ATTGGTAGGA
2521 AACAGGATAT GATAAAGCTG TTAATCGATC ATGGAGCTGA TGTAAACTCT TTAACTATTG
2581 CTAAAGATAA TCTTATTAAA AAAAAATAAT ATCACGTTTA GTAATATTAA AATATATTAA
2641 TAACTCTATT ACTAATAACT CCAGTGGATA TGAACATAAT ACGAAGTTTA TACATTCTCA
2701 TCAAAATCTT ATTGACATCA AGTTAGATTG TGAAAATGAG ATTATGAAAT TAAGGAATAC
2761 AAAAATAGGA TGTAAGAACT TACTAGAATG TTTTATCAAT AATGATATGA ATACAGTATC
2821 TAGGGCTATA AACAATGAAA CGATTAAAAA TTATAAAAAT CATTTCCCTA TATATAATAC
2881 GCTCATAGAA AAATTCATTT CTGAAAGTAT ACTAAGACAC GAATTATTGG ATGGAGTTAT
2941 AAATTCTTTT CAAGGATTCA ATAATAAAAT GCCTTACGAG ATTCAGTACA TTATACTGGA
3001 GAATCTTAAT AACCATGAAC TAAAAAAAAT TTTAGATAAT ATACATTAAA AAGGTAAATA
3061 GATCATCTGT TATTATAAGC AAAGATGCTT GTTGCCAATA ATATACAACA GGTATTTGTT
3121 TTTATTTTTA ACTACATATT TGATGTTCAT TCTCTTTATA TAGTATACAC AGAAAATTCA
3181 TAATCCACTT AGAAATTTCTA GTTATCTAG
```

FIG. 16

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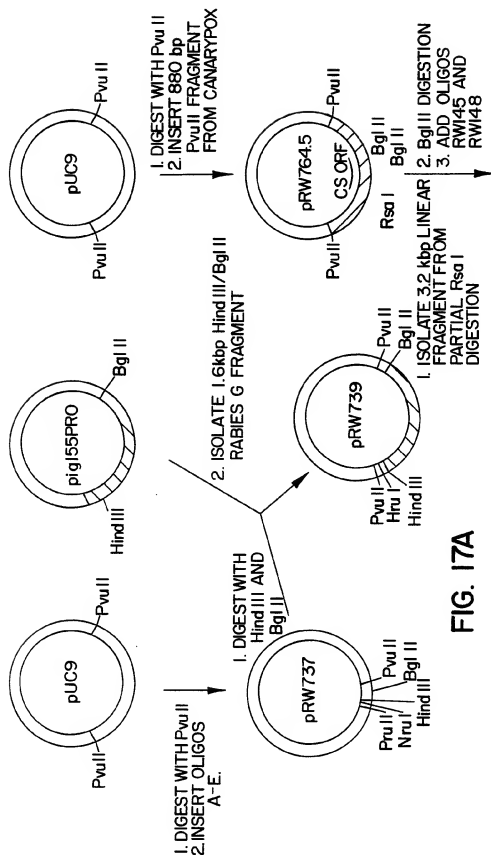
FIG. 17A

FIG. 17B
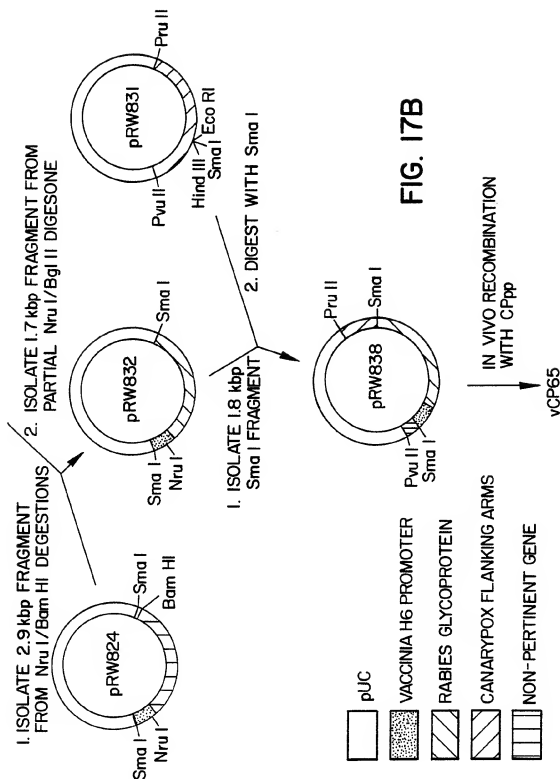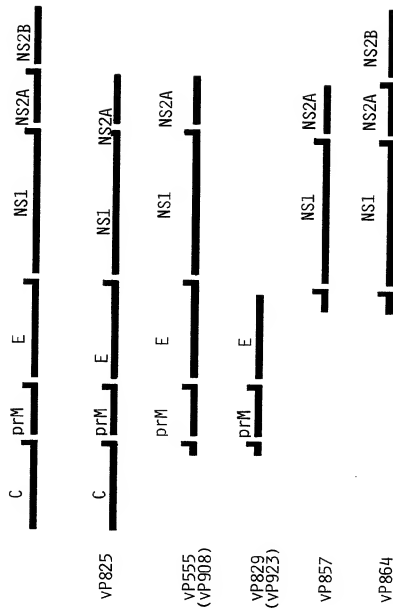
FIG. 18
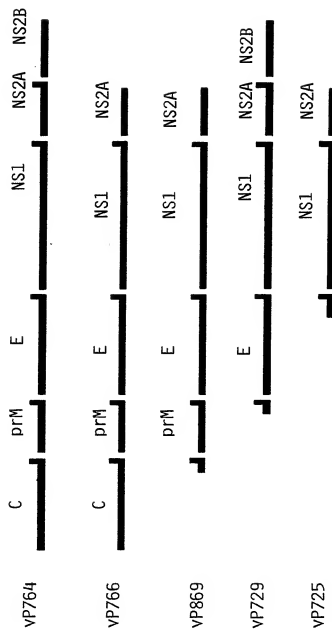
FIG. 19

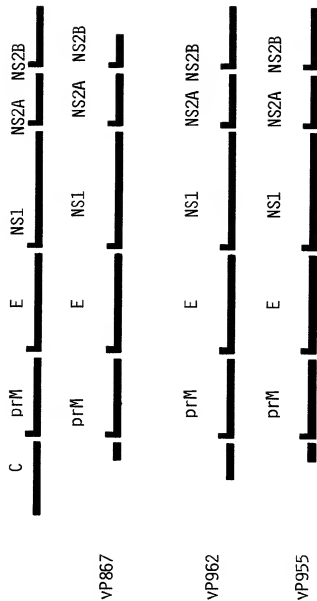FIG. 20

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              10         20         30         40         50         60
    1 GATATCTGTG GTCTATATAT ACTACACCCT ACCGATATTA ACCAACGAGT TTCTCACAAG
   61 AAAACTTGTT TAGTAGATAG AGATTCTTTG ATTGTGTTTA AAAGAAGTAC CAGTAAAAAG
  121 TGTGGCATAT GCATAGAAGA AATAAACAAA AAACATATTT CCGAACAGTA TTTTGGAATT
  181 CTCCCAAGTT GTAAACATAT TTTTTGCCTA TCATGTATAA GACGTTGGGG AGATACTACC
  241 AGAAATACAG ATACTGAAAA TACGTGTCCT GAATGTAGAA TAGTTTTTCC TTTCATAATA
  301 CCCAGTAGGT ATTGGATAGA TAATAAATAT GATAAAAAAA TATTATATAA TAGATATAAG
  361 AAAATGATTT TTACAAAAAT AACCTATAAG AACAATAAAA ATATAATTAC ATTTACGGAA
  421 AATAGCTGGT TTTAGTTTAC CAACTTAGAG TAATTATCAT ATTGAATCTA TATTGTTTTT
  481 TAGTTATATA AAAACATGAT TAGCCCCCAA TCGGATGAAA ATATAAAAGA TGTTGAGAAT
  541 TTCGAATACA ACAAAAAGAG GAATCGTACG TTGTCCATAT CAAACATAT AAATAAAAAT
  601 TCAAAAGTAG TATTATACTG GATGTTTAGA GATCAACGTG TACAAGATAA TTGGGCTTTA
  661 ATTTACGCAC AACGATTAGC GTTAAAACTC AAAATACCTC TAAGAATATG CTTTTGTGTC
  721 GTGCCAAAAT TTCACACTAC TACTTCTAGA CACTTTATGT TTTTTAATAT CCGGTCTTAA
  781 AGAAGTCGCG GGAGAATGTA AAAGACTATG TATAGGGTTT TCATTGATAT ATGGCGTACC
  841 AAAAGTAATA ATTCCGTGTA TAGTAAAAAA ATACAGAGTC GGAGTAATCA TAACGGATTT
  901 CTTTCCATTA CGTGTTCCCG AAAGATTAAT GAAACAGACT GTAATATCTC TTCCAGATAA
  961 CATACCTTTT ATACAAGTAG ACGCTCATAA TATAGTACCT TGTTGGGAAG CTTCTGATAA
 1021 AGAAGAATAC GGTGCACGAA CTTTAAGAAA AAAGATATTT GATAAATTAT ATGAATATAT
 1081 GACAGAATTT CCTGTTGTTC GTAAACATCC ATACGGTCCA TTTCTATAT CTATTGCAAA
 1141 ACCCAAAAAT ATATCATTAG ACAAGACGGT ATTACCCGTA AAATGGGCAA CGCCTGGAAC
 1201 AAAAGCTGGA ATAATTGTTT TAAAAGAATT TATAAAAAAC AGATTACCGT CATACGACGC
 1261 GGATCATAAC AATCCTACGT GTGACGCTTT GAGTAACTTA TCTCCGTGGC TACATTTTGG
 1321 TCATGTATCC GCACAACGTG TTGCCTTAGA AGTATTAAAA TGTATACGAG AAAGCAAAAA
 1381 AAACGTTGAA ACGTTTATAG ATGAAATAAT TGTAAGAAGA GAACTATCGG ATAATTTTTG
 1441 TTACTATAAC AAACATTATG ATAGTATCCA GTCTACTCAT TCATGGGTTA GAAAAACATT
 1501 AACGAGATCAC ATTAATGATC CTAGAAAGTA TATATATTCC ATTAAACAAC TCGAAAAAGC
 1561 GGAAACTCAT GATCCTCTAT GGAACGCGTC ACAAATGCAG ATGGTGAGAG AAGGAAAAAT
 1621 GCATAGTTTT TTACGAATGT ATTGGGCTAA GAAGATACTT GAATGGACTA GAACACCTGA
 1681 AGACGCTTTG AGTTATAGTA TCTATTTGAA CAACAAGTAC GAACTAGACG GCACGGATCC
 1741 TAACGGATAC GTAGGTTGTA TGTGGTCTAT TTGCGGATTA CACGATAGAG CGTGGAAAGC
 1801 AAGACCGATA TTTGGAAAGA TAAGATATAT GAATTATGAG AGTTCTAAGA AGAAATTTGA
 1861 TGTTGCTGTA TTTATACAGA AATACAATTA AGATAAATAA TATACAAGCAT TGTAACCATC
 1921 GTCATCCGTT ATACGGGGAA TAATATTACC ATACAGTATT ATTAAATTTT CTTACAGAAGA
 1981 ATATAGATCG GTATTTATCG TTAGTTTATT TATATTTAT TAATATTTAT GATAAAAATTA AGATAATAAT
 2041 TACCTGTTAT GGAAATGACA AATTTAGTTA TATAATTTAT GATAATTATG GAATTACGAG GAAGAAAGTT
 2101 AATGAAAATCA AATAATTATG TAAATGCTAC TAGATTATGT GAATTACGAG GATAATGTAA AAAAAATAAA
 2161 TACGAACTGG AAAAAAATTAA GTGAATCTAA AATATTAGTC GATAATGTAA ATGAGGATTA TTGATCATAA
 2221 TGATAAAACT AACCAGTTAA AAACGGATAT GATTTATATAC GTTAAGAATA TTGATCATAA
 2281 AGGAAGAGAT ACTTGCGGTT ACTATGTACA CCAAGATCTG GTATCTTCTA TATCAAATTG
 2341 GATATCTCCG TTATTCGCCG TTAAGTAAA TAAAATTATT AACTATTATA TATGTAATGA
 2401 ATATGATATA CGACTTAGCG AAATGGAATC TGATATGACA GAAGTAATAG ATGTAGTTGA
 2461 TAAATTAGTA GGAGGATACA ATGATGAAAT AGCAGAAATA ATATATTTGT TTAATAAATT
 2521 TATAGAAAAA TATATTGCTA ACATATCGTT ATCAACTGAA TTATCTAGTA TATTAAATAA
 2581 TTTTATAAAT TTTATAAATT TTAATAAAAA ATACAATAAC GACATAAAGA TATTTAATCT
 2641 TTAATTCTTG ATCTGAAAAA CACATCTATA AAATGAACTA AATCAGAGTA AAAAGTTATT CGATAAAGAT
 2701 AATAATGAAT CGAACGATGA AAAATTGGAA ACAGAAGTTG ATAAGCTAAT TTTTTTCATC
 2761 TAAATAGTAT TATTTTATTG AAGTACGAAG TTTTACGTTA TATCTGATAA AGATAAAAAT AAAGGTCGAT
 2821 TTTTACTTTG TTAAATATCA AATATGTCAT TATCTGATAA AGATAACATC ACACACGGTG
 2881 ATTATCAACC ATCTAACGAA CAGATATTAC AAAAAATACG TCGGACTATG GAAAACGAAG
 2941 CTGATAGGCCT CAATAGAAGA AGCATTAAAG AAATTGTTGT AGATAACTGGA AAATGATAC ATTAAACGAT
 3001 ATCATCCTCA ACGAAGAAAT AGATAAAGTT CTAAACTGGA AAATGATAC ATTAAACGAT
 3061 TTAGATCATC TAAAATACAGA TGATAATTAT AAGGAAATCA TACAATGTCT GATTAGAGAA
 3121 TTTGCGTTTA AAAAGATCAA TTCTATTATG TATAGTTATG CTATGGTAAA ACTCAATTCA
 3181 GATAACGAAC ATTGAAAGAT AAAATTAAAG ATTATTTTAT AGAAACTATT CTTAAAGACA
 3241 AACGTGGTTA TAAACAAAAG CCATTCACCG GATTGGAAAC TAAAATACTA GATAGTATTA
 3301 TAAGATTTTA AAACATAAA ATTAATAGGT TTTTATAGAT TGACTTATTA TATACAATAT
 3361 GGATAAAAGA TAATATCGAA CTAGAAAGTT GAATGAGCGA TTCTTAATTT TATATTTATGA
 3421 TTCAATAGAA ATTATTGTCA TGTCGTGTAA TCATTTTATA AATATATCAG CGTTACTAGC
 3481 TAAGAAAAAC AAGGACTTTA ATGAATGGCT AAAGATAGAA TCATTTAGAG AAATAATAGA
 3541 TACTTTAGAT AAAAATTAATT ACGATCTAGG ACACGATAT TGTGAAGAAC TTACGGCGCA
 3601 TCACATTCCA GTGTAATTAT TGAGGTCAAA GCTAGTAACT TAATAGATGA CAGGACAGCT
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   1 TGTCTGGACT AACTGATTTC ATGGAACAAT TTTCATCAAA AATATCAGTT ATACCTAGTT
  61 CTACAAAGAC AGAACTTTGA TGTTATGTTT GTGTTTGTAT AGAAAATTTT GGGATACTAA
 121 CTGATATTTC TGAATATTTC TGAATATTTC ATGTTACTTA CTTACTCCTA TCTTAGACGA
 181 TAATAAAATT CGAGGCGTAA TATGTTTTTC CAAATATTTG AAATTCTTAT ACGTATCGGC
 241 GAAGAAAAGT AACATACTAT AAGTGTTTAG CAAGTAAGGT ATGTTAATGA TATTGGATTT
 301 AATTTCATTG ACAATACATA TGTCCAAACA TTCCACTCGT AATTATGTAC GGAACGACTT
 361 TAGTTAAATA CTTAGTCACA AAAAACTTAT GACTGTCATT ATCTGAAAAC GGTGATTCCC
 421 ATAAATCAGA ATACTTAATA TTAAATAGAA TGCTCGCTTC TGGAGGTTTC CGGATACTAG
 481 ATAACATATC TTCTGTATTA TAGTTTAATT CACTCATTTT ATTACATAAT ACAGTAACAT
 541 CTCCCGAAAC CAATGATGTT ATATTAGATT TACTTACATA CTTCTTGTAA CTATCATGAA
 601 TACGTTTGTT GATGTCTATA AAGAAGATGG ATGTATATTC TGTTCTAGAT AGCAAGTTCT
 661 TTAAGTTATT CTTTGTCTGT ATTACTATCA TCGTCTTCAT CATCGTCTAA AGGTAGCATT
 721 ATATAATAAA TCTAATAGTT GATTTCTCGA TCTATCAGTA CTCGCTTTCA ATAACATTTT
 781 TACTATAAGC ATAATAGAAG GCGGTGATAT CACTATATTT TTATCGGGTA TTCTTTAAGT
 841 AATTAGTTAG TTCGTAGAAT TTCGTAGAGA TAAAAGCCAA TTTGTTGTTG ATACTGCTTA
 901 CGTTACTCAT GTTTCTTGTT TCTGTTAATT AACAGGTATA CCCTTACAAT AAGTTTAATT
 961 AACTTTTAGG TTTTTGTGAA GAACTTTTAG CTTCTAGTTC CCTTATCCAT AATTGGGTCT
1021 TAGATCTAGA TTCTTCCCAT GTATAAAGGG GGACATACCC AAAATCTTTA AATGCTTTGT
1081 CCGTTTCTAT GTAAAATGTC GTACATTCCT TAATCAAAGT ATAAGGATTT AGTAAAGGCG
1141 TGTAAGAACA AATAGGTGAT AGTAATACTC TTAAACCTTT ATTAATATTA GCGATAAACC
1201 TTAAACACCA TAAAGGAAGA CATGTATTCC GTAGATCCAT CCCTAATTGA TTAAAGAAAT
1261 GCATGTTAAA ATCATGATAA TGTTCAGTAG GAGAGGTATC GTAACAGTAA TACACGTTAT
1321 TGCAGAGAGG ACTATGTTGA CCATTTTCTA TCATATTTCT TGCTGCTAAA ATATGCATCC
1381 AAGCTACGTT TCCTGCATAG ACTCTGCTAT GAAATACTTT ATCATCCGCA TATTTATACA
1441 TTTTCCTGCT TTTATACGAT CTTCTGTATA AAGTTTCTAG TACTGGACAG TATTCTCCGA
1501 AAACACGTAA TGGGCGTAGC GACAAGTGCA TAATCTAAGT CCTATATTAG ACATAGTACC
1561 GTTAGCTTCT AGTATATATT TTCAGATAA CTTGTTTACT AAGAGGATAA GCCTCTTTAT
1621 GGTTAGATTG ATAATACGTA TTCTCGTTTC CTCTTATCAT CGCATCTCCG GAGAAAGTTA
1681 GGACCTACCG CAGAATAACT ACTCGTATAT ACTAAGACTC TTACGCCGTT ATACAGACAA
1741 GAATCTACTA CGTTCTTCGT TCCGTGATA TTAACGTCCA TTATAGAGTC GTTAGTAAAC
1801 TTACCCGCTA CATCATTTAT CGAAGCAATA TGAATGACCA CATCTGCTGA TCTAAGCGCT
1861 TCGTCCAAAG TACTTTTATT TCTAACATCT CCAATACAGG GAACTATCTT TATTATATTA
1921 CATTTTTCTA CAAGATCTAG TAACCATTGG TCGATTCTAA TATCGTAAAC ACGAACTTCT
1981 TTTTAAAGAG GATTCGAACA AGATAAGATT ATTTATAATG TGTCTACCTA AAAATCCACA
2041 CCCTCCGGGTT ACCACGTATA CTAGTGTACG CATTTTGAGT ATTAACTATA TAAGACCAAA
2101 ATTATATTTT CATTTTCTGT TATATTATAC TATATAATAA AAACAAATAA ATATACGAAT
2161 ATTATAAGAA ATTTAGAACA GTTTGCGTTA GTATTGCCTT TTTTATTAAC GGCGTGTTCT
2221 TGTAATTGCC GTTTAGAATA GTCTTTATTT ACTTTAGATA ACTCTTCTAT CATAACCGTC
2281 TCCTTATTCC AATCTTCTTC AGAAGTACAT GAGTACTTAC CGAAGTTTAT CATCATAGAG
2341 ATTATATATG AAGAAA
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FIG. 22

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   1 AGCAAAAGCA GGGGATACAT AATGAACACT CAAATTCTAA TATTAGCCAC TTCGGCATTC
  61 TTCTATGTAC GTGCAGATAA AATCTGCCTA GGACATCATG CTGTGTCTAA TGGAACCAAA
 121 GTAGACACCC TTACTGAAAA AGGAATAGAA GTTGTCAATG CAACAGAAAC AGTTGAACAA
 181 ACAAACATCC CTAAGATCTG CTCAAAAGGA AAACAGACTA TTGACCTTGG TCAATGTGGA
 241 TTACTAGGGA CCGTTATTGG TCCTCCCCAA TGTGACCAAT TTCTTGAATT CTCTGCTAAT
 301 TTAATAGTTG AAAGAAGGGA AGGTAATGAC ATTTGTTATC CAGGCAAATT TGACAATGAA
 361 GAAACATTGA GAAAAATACT CAGAAAATCC GGAGGAATTA AAAAGGAGAA TATGGGATTC
 421 ACATATACCG GAGTGAGAAC CAATGGAGAG ACTAGCGCAT GTAGAAGGTC AAGATCTTCC
 481 TTTTATGCAG AGATGAAATG GCTTCTATCC AGCACAGACA ATGGGATATT TCCACAAATG
 541 ACAAAGTCCT ACAAGAACAC TAAGAAGGTA CCAGCTCTGA TAATCTGGGG AATCCACCAC
 601 TCAGGATCAA CTACTGAACA GACTAGATTA TATGGAAGTG GGAATAAATC GATAACAGTT
 661 TGGAGCTCCA AATACCAACA ATCTTTTGTC CCAAATCCTG GACCAAGGCC GCAAATCAAT
 721 GGACAATCAG GAAGAATTGA CTTTGACTGG CTGATGGTAG ATCCCAATGA TACTGTGACT
 781 TTCAGTTTTA ATGGGGCCTT TATAGCACCT GACCGCGCCA GTTTTCTAAG AGGTAAATCT
 841 CTAGGAATTC AAAGTGACGC ACAGCTTGAC AATAATTGTG AAGGTGAATG CTATCATATT
 901 GGAGGTACTA TAATTAGCAA CTTGCCCTTT CAAAACATTA ATAGTAGGGC AATCGGAAAA
 961 TGCCCCAGAT ACGTGAAGCA GAAGAGCTTA ATGCTAGCAA CAGGAATGAA AAATGTTCCT
1021 GAAGCTCCTG CACATAAACA ACTAACTCAT CACATGCGCA AAAAAAGAGG TTTATTTGGT
1081 GCAATAGCAG GATTCATTGA AAATGGGTGG AAGGGATTAA TAGACGGATG GTATGGATAT
1141 AAGCATCAGA ATGCACAAGG AGAAGGGACT GCTGCAGACT ATAGAGAAA AAACCAATCTGACT
1201 ATCAACCAAA TAACCGGGAA ATTGAACAGA CTAATAGAAA AAACCAACCA GCAATTCGAA
1261 CTAATAGATA ATGAGTTCAA TGAAATAGAA AAACAAATTG GCAATGTTAT TAACTGGACT
1321 AGAGATTCTA TCATCGAAGT ATGGTCATAT AATGCAGAAT TCCTCGTAGC AGTGGAGAAT
1381 CAACACACTA TTGATTTAAC TGACTCAGAA ATGAACAAAC TATATGAAAA GGTAAGAAGA
1441 CAACTGGAGAG AAAATGCTGA GGAAGATGGT AATGGCTGTT TTGAAATATT CCACCAATGT
1501 GACAATGATT GCATGGCCAG CATTAGAAAC AATACATATG ATCATAAAAA ATACAGAAAG
1561 GAGGCAATAC AAAACAGAAT TCAGATTGAT GCAGTAAAGT TGAGCAGCGG TTACAAAATA
1621 ATACTTTGGT TTAGCTTCGG GGCATCATGT TTCTTATTTC TTGCCATTGC AATGGTTCTT
1681 GCTTTCATAT GCATAAAAAA TGGAAACATG CGGTGCACTA TTTGTATATA AGTTTGAAAA
1741 AACACCCTTG TTTTCTACT
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FIG. 23

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   1 AGCAAAAGCA GGGGATATTT CTGTCAATCA TGAAGACAAC CATTATTTTG ATACTACTGA
  61 CCCATTGGGT CTACAGTCAA AACCCAACCA GTGGCAACAA CACAGCCACA CTATGTCTGG
 121 GACACCATGC AGTAGCAAAT GGAACATTGG TAAAAACAAT AACTGACGAC CAAATTGAGG
 181 TGACAAATGC TACTGAATTA GTTCAGAGCA CTTCAATAGG GAAAAATATGC AACAACCCAT
 241 ATAGGGTTCT AGATGGAAGA AACTGCACAT TAATAGATGC AATGCTAGGA GATCCCCACT
 301 GTGATGTTTT TCAGTATGGA AATTGGGACC TCTTCATAGA AAGAAGCAGC GCTTTCAGCA
 361 ATTGCTACCC ATATGACATC CCTGACTATG CATCGCTCCG GTCTATTGTG GCATCTTCAG
 421 GAACATTAGA ATTCACAGCA GAGGGATTCA CATGGACAGG TGTCACTCAA AACGGAAGAA
 481 GTGGCGCCTG CAGAAGGGGA TCAGCCGATA GTTTCTTTAG CCGACTGAAT TGGCTAACAG
 541 AATCTGGAAA TTCTTACCCC ACATTGAATG TAACAATGCC TAACAATAAC AATTTCGATA
 601 AACTATACAT CTGGGGGATC CATCACCCGA GCACAAACAA TGAGCAGACA AAATTGTATG
 661 TCCAAGAATT AGGGCGAGTA ACAGTCTCAA CAAAAAGAAG TCAACAAACA ATAATCCCCA
 721 ACATCGGATC TAGACCGGGG GTCAGGGGTC AATCAGGCAG GATAAGCATA TATTGGACCA
 781 TTGTGAAACC TGGAGATATC CTAATGATAA ACAGTAATGG CAACTTAGTT GCACCGGCGG
 841 GATATTTCAA AATGCGAACA GGAAAAAGCT CTATAATGAG ATCAGATGCA CCCATAGACA
 901 CTTGTGTGTC CGAGTGTATT ACACCAAATG GAAGCATCCC CAACGACAAA CCATTCAAA
 961 ATGTGAACAA AGTTACATAT GGAAAATGCC CCAAGTATAT CAAGCAGAAT ACTTTGAAGC
1021 TGGCCACTGG GATGAGGAAT GTACCAGAAA AGCAAATCAG AGGAATCTTT GGAGCAATAG
1081 CGGGATTCAT AGAAAATGGC TGGGAGGGAA TGGTTGATGG GTGGTATGGA TTCCGATATC
1141 AGAATTCGGA AGGAACAGGA CAAGCTGCAG ATCTAAAGAG CACTCAAGCA GCCATCGACC
1201 AGATCAATGG AAAATTGAAC AGAGTGATTG AGAGAACCAA TGAGAAATTC CATCAAATAG
1261 AGAAGGAATT CTCAGAAGTA GAAGGGAGAA TCCAGGACTT GGAGAAGTAT GTAGAAGACA
1321 CCAAAATAGA CCTATGGTCC TACAATGCAG AGTTACTGGT GGCTCTAGAA AATCAACATA
1381 CGATTGACTT AACAGATGCA GGATGAATA AATTATTCGA GAAGACTAGG CGCCAGTTAA
1441 GGAGAAACGC GGAAGACATG GGGGGTGGAT GTTTCAAGAT TTATCACAAA TGTGATAATG
1501 CATGCATTGG ATCAATAAGA AATGGGACAT ATGACCATTA CATATACAGA GATGAAGCAT
1561 TAAACAACCG ATTTCAAATT AAAGGTGTTG AGTTGAAATC AGGCTACAAA GATTGGATAC
1621 TGTGGGATTC ATTCGCCATA TCATGCTTCT TAATTTGCGT TGTTCTATTG GGTTTCATTA
1681 TGTGGGCTTG CCAAAAAGGC AACATCAGAT GCAACATTTG CATTTGAGTA AACTGATAGT
1741 TAAAAACACC CTTGTTTCTA CT
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FIG. 24

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   1 AGCAAAAGCA GGGGATATTT CTGTCAATCA TGAAGACAAC CATTATTTTG ATACTACTGA
  61 CCCATTGGGT CTACAGTCAA AACCCAACCA GTGGCAACAA CACAGCCACA TTATGTCTGG
 121 GACACCATGC AGTAGCAAAT GGAACATTGG TAAAAACAAT AACTGATGAC CAAATTGAGG
 181 TGACAAATGC TACTGAATTA GTTCAAGACA TTTCAATAGG GAAAATATGC AACAATCCAT
 241 ATAGGGTTCT AGATGGAAGA AATTGCACAT TAATAGATGC AATGCTAGGA GACCCCCACT
 301 GTGATGTTTT TCNGTATGAG AATTGGGACC TCTTCATAGA AAGAAGCAGC GCTTTCAGCA
 361 ATTGCTACCC ATATGACATC CCTGACTATG CATCGCTCCG GTCCATTGTA GCATCCTCAG
 421 GAACATTAGA ATTCACAGCA GAGGGATTCA CATGGACAGG TGTCACTCAA AACGGAAGAA
 481 GTGGAGCCTG CAAAAGGGGA TCAGCCGATA GTTTCTTTAG CCGACTGAAT TGGCTAACAA
 541 AATCTGGAAA TTCTTACCCC ATATTGAATG TGACAATGCC TAACAATAAA AATTTCGATA
 601 AACTATACAT CTGGGGGATT CATCACCCGA GCTCAAACAA AGAGCAGACA AAATTGTATA
 661 TCCAAGAATC AGGACGAGTA ACAGTCTCAA CAGAAAGAAG TCAACAAACA GTAATCCCTA
 721 ACATCGGATC TAGACCGTGG GTCAGGGGTC AATCAGGCAG GATAAGCATA TACTGGACCA
 781 TTGTAAAACC TGGAGATATT CTAACGATAA ACAGTAATGG CAACTTAGTT GCACCGCGGG
 841 GATATTTTAA ATTGAGAACA GGGAAAAGCT CTGTAATGAG ATCAGATGCA CCCATAGACA
 901 CTTGTGTGTC TGAATGTATT ACACCAAATG GAAGCATCCC CAACGACAAA CCATTTCAAA
 961 ATGTGAACAA AGTTACATAT GGAAAATGCC CCAAGTATAT CAGGCAAAAC ACTTTAAAGC
1021 TGGCCACCGG GATGAGGAAT GTACCAGAAA AGCAAATCAG AGGAATCTTT GGAGCAATAG
1081 CGGGATTCAT AGAAAACGGC TGGGAAGGAA TGGTTGATGG GTGGTATGGA TTCCGGATATC
1141 AAAACTCGGA AGGAACAGGA CAAGTGCAG ATCTAAAAGAG CACTCAAGCA GCCATCGACC
1201 AGATCAATGG AAAATTAAAC AGAGTGATTG AAAGGACCAA TGAGAAATTC CATCAAATAG
1261 AGAAGGAATT CTCAGAAGTA GAAGGGAGAA TCCAGGATTT GGAGAAGTAT GTAGAAGACA
1321 CCAAAATAGA CCTATGGTCC TACAATGCAG AATTGCTGGT GGCTCTAGAA AATCAACATA
1381 CAATTGACTT ACAGATGCA GAAATGAATA AATTATTCGA GAAGACTAGG CGCCAGTTAA
1441 GAGAAAACGC GGAAGACATG GGAGGTGGAT GTTTCAAGAT TTACCACAAA TGTGATAATG
1501 CATGCATTGG ATCAATAAGA AATGGGACAT ATGACCATTA CATATACAGA GATGAAGCAT
1561 TAAACAACCG ATTTCAAATC AAAGGTGTTG AGTTGAAATC AGGCTACAAA GATTGGATAC
1621 TGTGGATTTC ATTCGCCATA TCATGCTTCT TAATTTGCGT TGTTCTATTG GGTTTCATTA
1681 TGTGGGCTTG CCAAAAAGGC AACATCAGAT GCAACATTTG CATTTGAGTA AACTGATAGT
1741 TAAAAACACC CTTGTTTCTA CT
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FIG. 25

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   1 CCCCAGCTCA GACGATCTGT CAAGATGGAA GGTCCAACGC ACCCAAAACC CTCTAAAGAT
  61 AAGACTTTCT CGTGGGACCT AATGATTCTG GTGGGGGTCT TACTAAGACT GGACGTGGGA
 121 ATGGCCAATC CTAGTCCGCA CCAAATATAT AATGTAACTT GGACAATAAC CAACCTTGTA
 181 ACTGGAACAA AGGCTAATGC CACCTCCATG TTGGGAACCC TGACAGACGC CTTCCCTACC
 241 ATGTATTTTG ACTTATGTGA TATAATAGGA AATACATGGA ACCCTTCAGA TCAAGAACCA
 301 TTCCCAGGGT ATGGATGTGA TCAGCCTATG AGGAGGTGGC GACAGAGAAA CACACCCTTT
 361 TATGTCTGTC CAGGACATGC CAACCGGAAG CAATGTGGGG GGCCACAGGA TGGGTTCTGC
 421 GCTGTATGGG GTTGCGAGAC CACCCGGGGAA ACCTATTGGA GACCCACCTC CTCATGGGAC
 481 TACATCACAG TAAAAAAAGG GGTTACTCAG GGAATATATC AATGTAGTGG AGGTGGTTGG
 541 TGTGGGGCCCT GTTACGATAA AGCTGTTCAC TCCTCGACAA CGGGAGCTAG TGAAGGGGGC
 601 CGGTGCAACC CCTTGATCTT GCAATTTACC CAAAAAGGGAA GACAAACATC TTGGGATGGA
 661 CCTAAGTCAT GGGGGCTACG ACTATACCGT TCAGGATATG ACCCTATAGC CCTGTTCTCG
 721 GTATCCCGGC AAGTAATGAC CATTACGCCG CCTCAAGGTCA CTGGACCAAA TCTAGTCCTG
 781 CCTGATCAAA AACCCCCATC CAGGCAATCT CAAATAGAGT CCCGAGTAAC ACCTCACCAT
 841 TCCCAAGGCA ACGGAGGCAC CCCAGGTGTA ACTCTTGTTA ATGCCTCCAT TGCCCCTCTA
 901 CGTACCCCTG TCACCCCCGC AAGTCCCAAA CGTATAGGGA CCGGAAATAG GTTAATAAAT
 961 TTAGTGCAAG GGACATACCT AGCCTTAAAT GCCACCGACC CCAACAAAAC TAAAGACTGT
1021 TGGCTCTGCC TGGTTTCTCG ACCACCTTAT TACGAAGGGA TTGCAATCTT AGGTAACTAC
1081 AGCAACCAAA CAAACCCCTC CCCATCCTGC CTATCTACTC CGCAACATAA GCTAACTATA
1141 TCTGAGGTGT CAGGGCAAGG ACTGTGCATA GGGACTGTTC CTAAGACCCA CCAGGCTTTG
1201 TGCAATAAGA CACAACAGGG ACATACAGGG GCTCATCATC TAGCCGCCCC CAATGGCACC
1261 TATTGGGGCCT GTAACACTGG ACTCACCCCA TGCATTTCCA TGGCAGTGCT CAATTGGACC
1321 TCTGATTTTT GTGTCTTAAT CGAATTATGG CCCAGAGTGA CCTACCATCA ACCCGAATAC
1381 ATTTACACAC ATTTCGACAA AGCTGTCAGG TTCCGAAGAG AACCAATATC ACTAACCGTT
1441 GCCCTTATAA TGGGAGGACT CACTGTAGGG GGCATAGCCG CGGGGGTCGG AACAGGGACT
1501 AAAGCCCTCC TTGAAACAGC CCAGTTCAGA CAACTACAAA TGGCTATGCA CGCAGACATC
1561 CAGGCCCTAG AAGAGTCAAT TAGTGCCTTA GAAAAATCCC TGACCTCCCT CTCCGAGGTA
1621 GTCTTACAAA ATAGACGGGG CCTAGATATT CTGTTCTTAC AAAAGGGAGG GCTCTGTGCC
1681 GCCTTAAAGG AAGAATGCTG CTTCTATGCA GATCACACCG GACTCGTCAG AGACAATATG
1741 GCTAAATTAA GAGAAAGACT GAAACAGCGA CAACAACTGT TTGACTCCCA ACAGGGATGG
1801 TTTGAAGGAT GGTCAACAA GTCCCCCTGG TTTGAACCTT TAATTTCCTC CATTATAGGC
1861 CCCTTACTAA TCCTACTCCT AATTCTCCTC TTCGGCCCAT GCATCCTTAA CCGATTAGTG
1921 CAATTCGTAA AAGACAGAAT ATCTGTGGTA CAAGCCTTAA TTTTAACCCA ACAGTACCAA
1981 CAGATACAGC AATATGATCC GGACCGACCA TGATTTCCAA TTAAATGTAT GATTCCATTT
2041 AGTCCCTAGA AGAAGGGGGG AATGAAAGAC CCCCCCCACC CCAAAACTTA GCCAGCTACT
2101 GCAGTGATGC CATTTCACAA GACATGGAAA ATTACCCAGG CATGTTCCCG TGAGATATAA
2161 GGAAGTTAGA AGCTAAAACA GGATATCTGT GGTTAGACAC CTAGGCCCCG GCTTGAGGCC
2211 AAGAACAGTT AAGCCCCGGA TATAGCTGAA ACAGCAGAAG TTTCAAGGCC ACTGCCAGCA
2281 GTCTCCAGGC TCCCCAGTTG ACCAGAGTTC GACCTTCCGC CTCATTTAAA CTAACCAATC
2341 CCCACGCCTC TCGCTTCTGT ACGCGCGCTT TTTGCTATAA AACGAGCCAT CAGCCCACCA
2401 CCAGGCGCGC AAGTCTTTGC AGAGACTTGA CGCCCCGGGC CCACGCCTCT CGCTTCTGTA
2461 CGCGCGCTTT TTGCTATAAA ACGAGCCATC AGCCCACCA
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FIG. 26

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   1 CTGCAGTGGT GTCATTTCAC AAGGCATGGA AAATTACTCA AGTATGTTCC CATGAGATAT
  61 AAGGAAGTTA GAGGCTAAAA CAGGATATCT GTGGTTAAGC ACCTGGGCCC CGGCTTGAGG
 121 CCAAGAACAG TTAAACCCCG GATATAGCTG AAACAGCAGA AGTTTCAAGG CCGCTGCCAG
 181 CAGTCTCCAG GCTCCCCAGT TGACCAGAGT TCGACCTTCC GCCTCATTTA AACTAACCAA
 241 TGCCCACGCC TCTCGGCTTCT GTGCGGCGGC TTTCTGCTAT AAAACGAGCC ATCAGCCCCC
 301 AACGGCGCGC AAGTCTTTGC TGAGACTTGA CCGCCCCGGG TACCCGTGTA CGAATAAACC
 361 TCTTGCTGAT TGCATCTGAC TGTGGTCTCG GTGTTCCGTG GGCACGGGGT CTCATCGCCG
 421 AGGAAGACCT AGTTCGGGGG TCTTTCATTT AGGGGCTCGT CCGGGATAGA GACCCCCAAC
 481 CCCCGGGACC ACCGACCCAC CACCATCAGG AGGTAAGCTG GCCGGCGACC ATACCTGTTG
 541 TCCTTGTATA AGTGTCTCTG TCAATTGATG TGATTTTGGC GGTGGGATCG AAGGAGCTGA
 601 CGAGCTCGTA CTTCGCCCCG GCAACCCTGG AAGACATTCC ACGGGTGTCT GATGTCTGGA
 661 GGCCTCAGTG GGACAGCCAT TGGGGCTCAT CTGTTTGGGG TCTCACCTGA ATACAGGGTG
 721 TTGATCGGAG ACGAGGGGAGC CGGACCCTGA AGGTCTCTTT CTGAGGTTTC ATTTTCGGTT
 781 TGGTACCAAA GAGCGCGGGC ACGTCTTGTC ATTTTTGTC TGGTTGCGTC TTTTCTTGTC
 841 CCTTGTCTAA CCTTTTTAAT TGCAGAAACC GTCATGGGCC AAACTATAAC TACCCCCTTA
 901 AGCCTCACCC TTGATCACTG GTCTGAAGTC CGGGCACGAG CCCATAATCA AGGTGTCGAG
 961 GTCCGGAAAA AGAAATGGAT TACCTTATGT GAGGCCGAAT GGGTGATGAT GAATGTGGGC
1021 TGGCCCCGAG AAGGAACTTT TTCTCTTGAT AGCATTTCCC AGGTTGAAAA GAAGATCTTC
1081 GCCCCGGGAC CATATGGACA CCCCGACCAA GTTCCTTACA TTACTACATG GAGATCCTTA
1141 GCCACAGACC CCCCTTCGTG GGTTCGTCCG TTCCTACCCC CTCCCAAACC TCCCACACCC
1201 CTCCCTCAAC CTCTTTCGCC GCAGCCCTCG GCCCCTCTTA CCTCTTCCCT CTACCCCGTT
1261 CTCCCCAAGC CAGACCCCCC CAAACCGCCT GTGTTACCGC CTGATCCTTC TTCCCCTTTA
1321 ATTGATCTCT TAACAGAAGA GCCACTCTCC TATCTCGGGGG GTCACGGGCC ACCGCCATCA
1381 GGTCCTAGGA CCCCAACCGC TTCCCCGATT GCAAGCCGGC TAAGGGAACG ACGAGAAAAC
1441 CCTGCTGAAA AATCGCAAGC CCTCCCCTTG AGGGAAGGCC CCAACAACCG ACCCCAGTAT
1501 TGGCCATTCT CAGCTTCAGA CTTGTATAAC TGGAAGTCGC ATAACCCCCC TTTCTCCCAA
1561 GATCCAGTGG CCCTAACTAA CCTAATTGAG TGCATTTTAG TGACGCATCA ACCAACCTGG
1621 GACGACTGCG AGCAGCTCTT GCAGGCACTC CTGACAGGCG AAGAAAGGCA AAGGGTCCTT
1681 CTTGAGGCCC GAAAGCAGGT TCCAGGCGAG GACGGACGGC CAACCAACT ACCCAATGTC
1741 ATTGACGGAGA CTTTCCCCTT GACCCGTCCC AACTGGGATT TTGCTACGCC GGCAGGTAGG
1801 GAGCACCTAC GCCTTTATCG CCAGTTGCTA TTAGCGGGTC TCCGCGGGGC TGCAAGACGC
1861 CCCACTAATT TGGCACAGGT AAAGCAGGTT GTACAAGGAA AAGAGGAAAC GCCAGCAGCA
1921 TTTTTAGAAA GATTAAAAGA GGCTTATAGA ATGTACACTC CCTATGACCC TGAGGACCCA
1981 GGGCAAGCGG CTAGTGTTAT CCTATCCTTT ATATACCAGT CTAGCCCAGA TATAAGAAAT
2041 AAGTTACAAA GGCTAGAAGG CCTACAAGGG TTCACCCTAT CTGATCTGCT AAAAGAGGCA
2101 GAAAAGATAT ACAACAAAAG GGAGACCCCA GAGGAAAGGG AAGAAAGATT ATGGCAGCGA
2161 CAGGAAGAAA GAGATAAAGA GCGCCACAAG GAGATGACTA AAGTTCTGGC CACAGTAGTT
2221 GCTCAGAATA GAGATAAGGA TAGAGAAGAA AGTAAACTGA GGGATCAAAG GAAAATACCT
2281 CTGGGGAAAG ACCAGTGTGC CTATTGCAAG GAAAAGGGGC ATTGGGTTCG CGATTGCCCC
2341 AAACGACCCA GGAAAAAACC CGCCAACTCC ACTCTCCTCA ACTTAGAGAA TTAGGAGAGT
2401 CAGGGCCAGG ACCCCCCCCC CTGAGCCCAG GATAACCTTA AAAATAGGGG GGCAACCGGT
2461 GACTTTTCTG GTGGACACGG GGCCCCAGCA CTCAGTACTG ACTCGACCAG ATGGACCTCT
2521 CAGTGACCGC ACAGCCCTGG TGCAAGGAGC CACGGGAAGC AAAAACTACC GGTGGACCAC
2581 CGACAGGAGG GTACAACTGG CAACCGGTAA GGTGACTCAT TCTTTTTTAT ATGTACCTGA
2641 ATGTCCCTAC CCGTTATTAG GGAGAGACCT ATTAACTAAA CTTAAGGCCC AAATCCATTT
2701 TACCGGAGAA GGGGCTAATG TGTTGGGGCC CAGGGGTTTA CCCCTACAAG TCCTTTACTTT
2761 ACAATTAGAA GAGGAGTATC GGCTATTTGA GGCCAGAAGT ACACAAAAAC AGGAGATGGA
2821 CACTTGGCTT AAAAACTTTC CCCAGGCGTG GGCAGAAACA GGAGGTATGG GAATGGCTCA
2881 TTGTCAAGCC CCCGTTCTCA TTCAACTTAA GGCTACTGCC ACTCCAATCT CCATCCGACA
2941 GTATCCTATG CCCCCATGAAG CTCAAGCCCT GCCAGTCCCC ATGGAATACA CCCTTATTAC CTGTTAAGAA
3001 TCAAGGCATC CTCAAGCCCT GCCAGTCCCC ATGGAATACA CCCTTATTAC CTGTTAAGAA
3061 GCCAGGGACC GAGGATTACA GACCAGTGCA GGACTTAAGA GAAGTAAACA AAAGAGTAGA
3121 AGACATCCAT CCTACTGTGC CAAATCCATA TAACCTCCTT AGCACCCTCC CGCCGTCTCA
3181 CCCTTGGTAC ACTGTCCTAG ATTTAAAGGA CGCTTTTTCC TGCCTGCGAC TACACTCTGA
3241 GAGTCAGTTA CTTTTTGCAT TTGAATGGAG AGATCCAGAA ATAGGACGT CAGGGCAACT
3301 AACCTGGACA CGCCTTCCTC AGGGGTTCAA GAATAGCCCC ACCCTATTTG ATGAGGCCCT
3361 GCACTCAGAC CTGGCCGATT TCAGGGTAAG GTACCCGGCT CTAGTCCTTC TACAATATGT
3421 AGATGACCTC TTGCTGGCTG CGGCAACCAG CAGTGAATGC CTGGAAGGGA CTAAGGCACT
3481 CCTTGAGACT TTGGGCAATA AGGGGTACCG AGCCTCTGGA AAGAAGGCCC AAATTTGCCT
3541 GCAAGAAGTC ACATACCTGG GGTACTCTTT AAAAGATGGC CAAAGGTGGC TTACCAAAGC
3601 TCGGAAAGAA GCCATCCTAT CCATCCCTGT GCCTAAAAAC CCACGACAAG TGAGAGAGTT
3661 CCTTGGAACT GCAG
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   1 ATGATGACAC GTCTACATTT TTGGTGGTGT GGAATCTTTG CGGTCCTGAA ATATCTGGTA
  61 TGTACTTCAA GCCTTACGAC CACGCCAAAA ACAACTACGG TTTATGTGAA GGGATTTAAT
 121 ATACCTCCAC TACGCTACAA TTATACTCAA GCCAGAATCG TGCCAAAAAT TCCCCAGGCG
 181 ATGGATCCGA AGATAACAGC TGAAGTACGT TATGTAACAT CAATGGATTC ATGTGGGATG
 241 GTGGCATTGA TATCAGAGCC GGATATAGAC GCTACTATTC GAACCATACA ACTATCTCAA
 301 AAAAAAACAT ATAACGCGAC TATAAGTTGG TTTAAGGTAA CCCAGGGTTG TGAATACCCT
 361 ATGTTTCTTA TGGATATGAG ACTTTGTGAT CCTAAACGGG AATTTGGAAT ATGTGCTTTA
 421 CGGTCGCCTT CATATTGGTT GGAACCTTTA ACAAAGTATA TGTTCCTAAC AGACGATGAA
 481 CTGGGTTTGA TTATGATGGC CCCGGCCCAA TTTAATCAAG GACAATATCG AAGAGTTATA
 541 ACCATCGATG GTTCCATGTT TTATACAGAT TTTATGGCTAC AACTATCTCC AACGCCATGT
 601 TGGTTCGCAA AACCCGATAG ATACGAAGAG ATTCTACATG AATGGTGTCG AAATGTTAAA
 661 ACTATTGGCC TTGATGGAGC TCGTGATTAC CACTATTATT GGGTACCCTA TAACCCACAA
 721 CCTCACCATA AAGCCGTACT CTTTATATTGG TATCGGACTC ATGGCCGAGA ACCCCCAGTA
 781 AGATTCCAAG AGGCCATTCG ATATGATCGT CCCGCCATAC CGTCTGGGAG TGAGGATTCG
 841 AAACGGTCCA ACGACTCTAG AGGAGAATCG AGTGGACCCA ATTGGATAGA CATTGAAAAT
 901 TACACTCCTA AAAATAATGT GCCTATTATA ATATCTGACG ATGACGTTCC TACAGCCCCT
 961 CCCAAGGGCA TGAATAATCA GTCAGTAGTG ATACCCGCAA TCGTACTAAG TTGTCTTATA
1021 ATAGCACTGA TTCTAGGAGT GATATATTAT ATTTTGAGGG TAAAGAGGTC TCGATCAACT
1081 GCATATCAAC AACTTCCTAT AATACATACA ACTCACCATC CTTAA
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FIG. 28

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   1 GAGCTCGAAA ATAGAATTGA TCAGTAATCA TGAAGGCTTT TTTAGTTACT TGCTTAAGCT
  61 TTGCAGTCTT TTCATCTTCT GTATGTGTGA ATATCAACAT CTTGCAGCAA ATTGGATATA
 121 TCAAGCAACA AGTCAGGCAA CTAAGCTATT ACTCACAAAG TTCAAGCTCC TACATAGTGG
 181 TCAAGCTTTT ACCGAATATC CAACCCATTG ATAACAGCTG TGAATTTAAG AGTGTAACTC
 241 AATACAATAA GACCTTGAGT AATTTGCTTC TTCCAATTGC AGAAAACATA AACAATATTG
 301 CATCGCCCTC ATCTGGGTCA AGACGGCATA AAAGGTTTGC TGGTATTGCT ATTGGCATTG
 361 CTGCGCTCGG TGTTGCGACC GCAGCACAAG TAACTGCCGC TGTCTCATTA GTTCAAGGAC
 421 AGACAAATGC ACGTGCAATA GCGGCGATGA AAAATTCAAT ACAAGCAACT AATCGAGCAG
 481 TCTTCGAAGT GAAGGAAGGC ACTCAACAGT TAGCTATAGC GGTACAAGCA ATAAGAGACC
 541 ACATCAATAC TATTATGAAC ACCCAATTGA ACAATATGTC TTGTCAGATC CTTGATAACC
 601 AGCTTGCAAC TTTCCTAGGA TTATACCTAA CAGAATTAAC AACAGTGTTT CAGCCACAAT
 661 TAATTAATCC GGCATTGTCA CCGATTAGTA TACAAGCCTT GAGGTCTTTG CTTGGAAGTA
 721 TGACGCCTGC AGTGGTCCAA GCAACATTAT CTACTTCAAT CTCTACTGCT GAAATACTAA
 781 GTGCCGGTCT AATGGAGGGT CAGATTGTTT CTGTTCTGCT AGATGAGATG CAGATGATAG
 841 TTAAGATAAA TATTCCAACT ATTGTCACAC AATCAAATGC ATTGGTGATT GACTTCTACT
 901 CAATTTCGAG CTTTATTAAT AATCAGGAAT CCATAATCCA ATTGCCAGAC AGAATCTTGG
 961 AGATCGGGAA TGAACAATGG AGCTATCCAG CTAAAAATTG TAAGTTGACA AGACACCACA
1021 TATTCTGCCA ATACAATGAG GCAGAGAGGC TGAGCCTAGA ATCAAAACTA TGCCTTGCAG
1081 GTAATATAAG TGCCTGTGTG TTCTCACCCA TAGCAGGGAG TTATATGAGG CGATTTACGG
1141 CACTGGATGG AACAATTGTT GCAAACTGTC GAAGTCTAAC GTGTCTATGC AAGAATCCAT
1201 CTTATCCTAT ATACCAACCT GACCATCATG CAGTCACGAC CATTGATCTA ACCGCATGTC
1261 AAACATTGTC CCTAGACGGA TTGGATTTCA GCATTGTCTC TCTAAGCAAC ATCACTTACG
1321 CTGAGAACCT TACCATTTCA TTGTCTCAGA CAATCAATAC TCAACCCATT GACATATCAA
1381 CTGAACTGAG TAAGGTTAAT GCATCCCTCC AAAATGCCGT TAAGTACATA AAGGAGAGCA
1441 ACCATCAGCT CCAATCTGTG AGTGTAAACT CCAAAATCGG AGCTATAATT GTAGCAGCCT
1501 TAGTTTTGAG CATTCTGTCA ATTATCATTT CGCTATTGTT TTGCTGCTGG GCTTACATTG
1561 CAACTAAAGA AATCAGAAGA ATCAACTTCA AAACAAATCA TATCAATACA ATATCAAGTA
1621 GTGTCGATGA TCTCATTAGG TACTAATCCT AACATTGTGA TTCATCCTGC ATTCAGAAAG
1681 GTACC
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FIG. 29

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   1 GAGCTCCAAT ACAACACAGA ACCCCAGCTG CCATCACAAC TGTTCTCTGG CCGCTCGAAA
  61 GATGGAGCCC TCAAAACTCT TCACAATGTC AGACAATGCC ACCTTTGCAC CTGGACCTTT
 121 TATCAATGCG GCAGACAAGA AGACGTTCGG AACCTGCTTC CGAATATTGG TACTGTCTGT
 181 ACAAGCTGTT ACCCTTATAT TAGTTATTGT CACTTTAGGT GAGCTTGTGA GGATGATCAA
 241 TGATCAAGGC TTGAGCAATC AGTTGTCTTC AATTGCAGAC AAGATAAGAG AGTCAGCTAC
 301 TATGATTGCA TCTGCTGTGG GAGTAATGAA TCAAGTTATT CACGGAGTAA CGGTATCCTT
 361 ACCCCTACAA ATTGAGGGAA ACCAAAATCA ATTGTTATCC ACACTTGCCA CAATCTGTAC
 421 AGGCAAAAAA CAAGTCTCAA ACTGCTCTAC AAACATCCCC TTAGTTAATG ACCTTAGGTT
 481 TATAAATGGG ATCAATAAAT TCATCATTGA AGATTATGCA ACTCATGATT TCTCTATCGG
 541 CCATCCACTC AACATGCCTA GCTTTATCCC AACTGCAACT TCACCCAATG GTTGCACAAG
 601 AATTCCATCC TTTTCTCTAG GTAAGACACA CTGGTGCTAA ACACATAATG TAATTAATGC
 661 CAACTGCAAG GATCATACTT CGTCTAACCA ATATATTTCC ATGGGGATAC TGTTCAGAC
 721 CGCGTCAGGG TATCCTATGT TCAAAACCTT AAAAATCCAA TATCTCAGTG CAGGGGTTAA
 781 TCGGAAAAGC TGCTCAATTG CAACAGTCCC TGATGGATGC GCAATGTACT GTTACGTCTC
 841 AACTCAACTT GAAACCGACG ACTATGCGGG GTCCAGCCCA CCTACCCAGA AACTTACCCT
 901 GTTATTCTAT AATGATACCG TCACAGAAAG GACAATATCT CCAACTGGTC TTGAAGGGAA
 961 TTGGGCTACT TTGGTTCCAG GAGTGGGGAG TGGAATATAT TTCGAGAATA AATTGATTTT
1021 TCCTGCATAT GGGGGTGTCT TGCCCAATAG TACACTCGGA GTTAAATCAG CAAGAGAATT
1081 TTTCCGGCCT GTTAATCCAT ATAATCCATG TTCAGGACCA CAACAAGATT TAGATCAGCG
1141 TGCTTTGAGA TCATACTTCC CAAGTTACTT CTCTAATCGA AGAGTACAGA GTGCATTTCT
1201 TGTCTGTGCC TGGAATCAGA TCCTAGTTAC AAATTGCGAG CTAGTTGTCC CCTCAAACAA
1261 TCAGACACTG ATGGGTGCAG AAGGAAGAGT TTTATTGATC AATAATCGAC TATTATATTA
1321 TCAGAGAAGT ACCAGCTGGT GGCCGTATGA ACTCCTCTAT GAGATATCAT TCACATTTAC
1381 AAACTCTGGT CAATCATCTG TGAATATGTC CTGGATACCT ATATATTCAT TCACTCGTCC
1441 TGGTTCAGGC AACTGCAGTG GTGAAAATGT GTGCCCAACT GCTTGTGTGT CAGGGGTTTA
1501 TCTTGATCCC TGGCCATTAA CTCCATATAG CCACCAATCA GGCATTAACC GAAATTTCTA
1561 TTTCACAGGC GCACTATTAA ATTCAAGCAC AACTAGAGTA AATCCTACCC TTTATGTCTC
1621 TGCCCTTAAT AATCTTAAAG TACTAGCCCC ATATGGTAAT CAGGGACTGT TTGCCTCGTA
1681 CACCACAACC ACCTGCTTTC AAGATACCGG TGATGCTAGT GTGTATTGTG TTTATATTAT
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1801 CATCACTTGA GTAATAGTGA ATGCGGTACC
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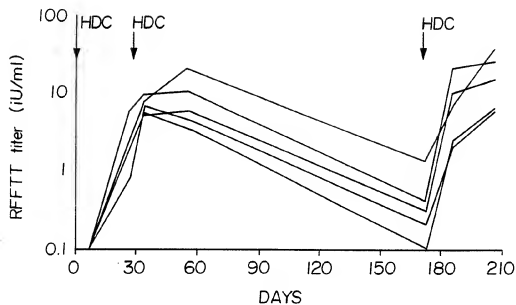
FIG. 30
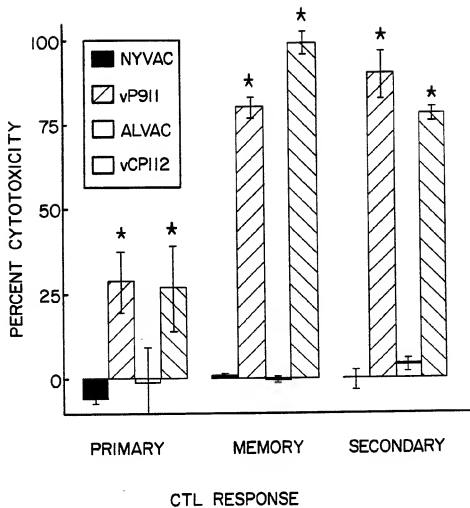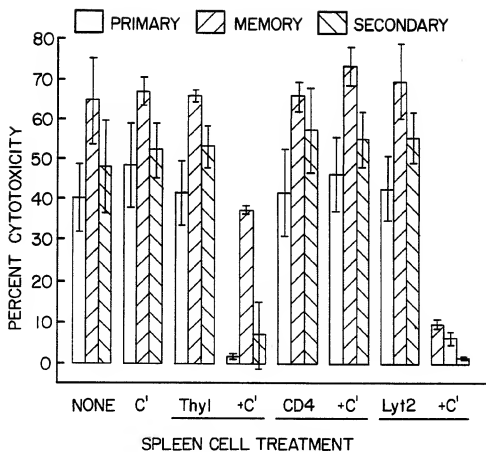
FIG. 31

FIG. 32

FIG. 33

FIG. 34

FIG. 35A
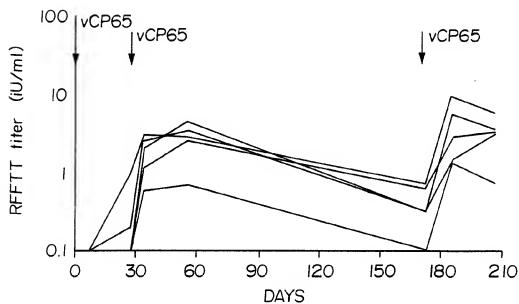

FIG. 35C

FIG. 35B

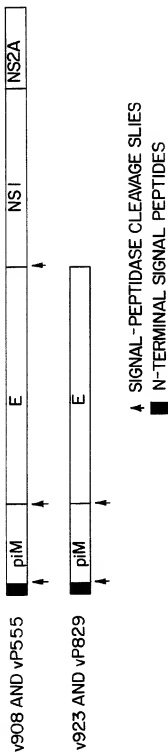FIG. 35D

FIG. 36
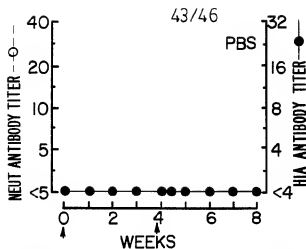
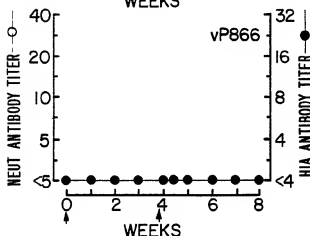↑ SIGNAL-PEPTIDASE CLEAVAGE SLIES
■ N-TERMINAL SIGNAL PEPTIDES

v908 AND vP555

v923 AND vP829
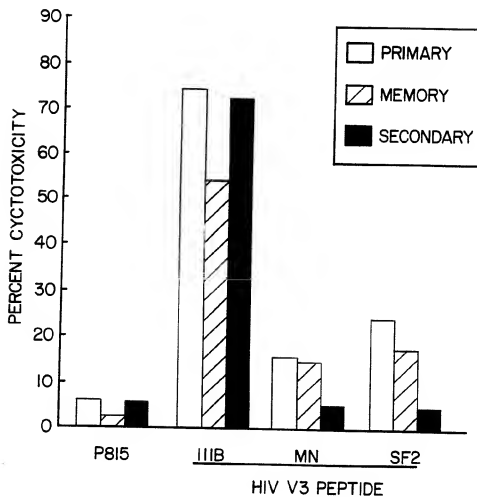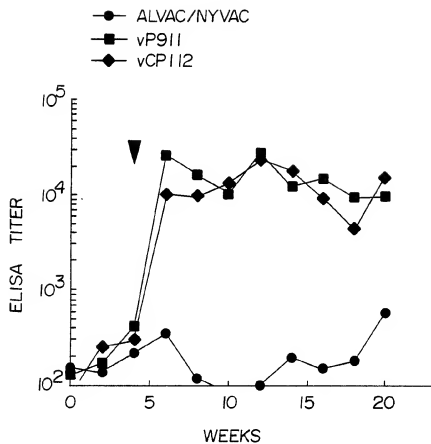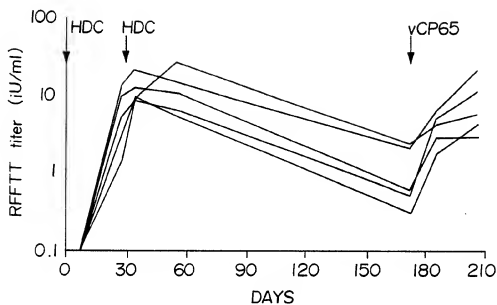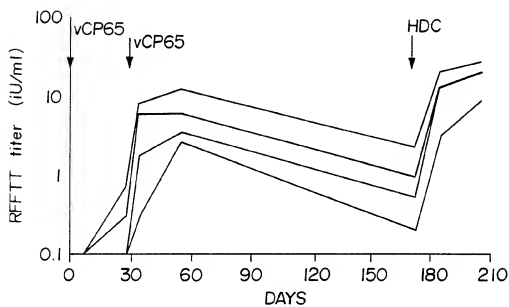
FIG. 37A

FIG. 37B

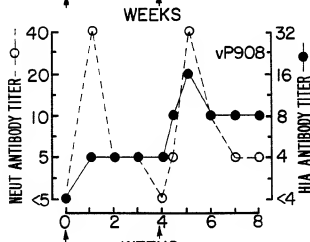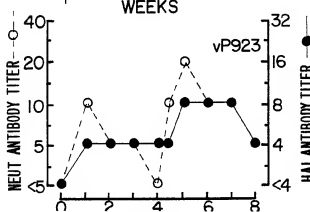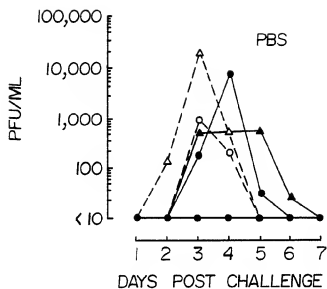FIG. 37C

FIG. 37D
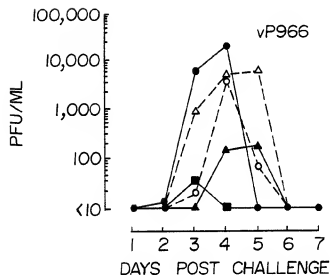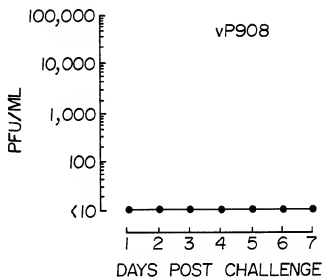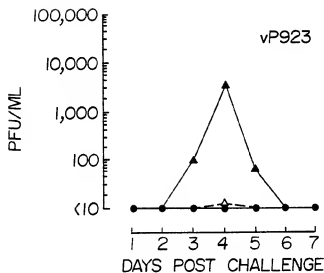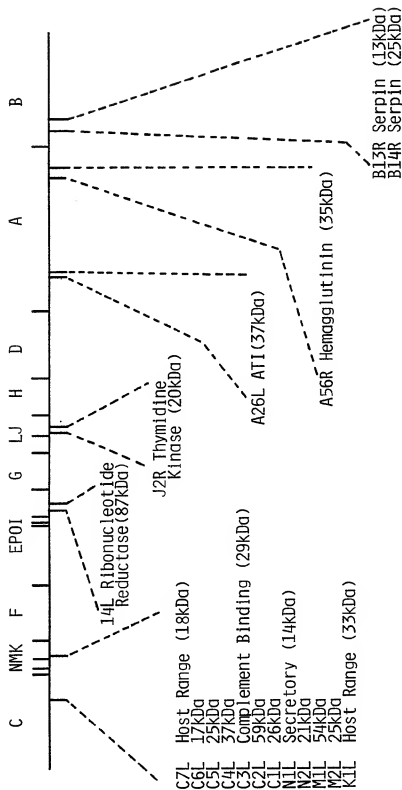
FIG. 38A

FIG. 38B

FIG. 38C

FIG. 38D

FIG. 39

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]
According to International Patent Classification (IPC) or to both National Classification and IPC
IPC (5): C12N 7/00, 15/00; C12P 21/06; A61K 39/12 US CL : 435/235.1, 69.1, 320.1; 424/89

II. FIELDS SEARCHED

Minimum Documentation Searched[4]	
Classification System	Classification Symbols
U.S.	435/235.1, 69.1, 320.1, 172.3; 424/89

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]
CA, BIOSIS, MEDLINE, WPI, APS search terms: poxvirus, vaccinia, attenuat?, deletion, insertion, vaccine, vector, avipox, fowlpox, canarypox, NYVAC, ALVAC

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]		
Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X/Y	Proceedings of the National Academy of Sciences USA, Volume 86, issued February 1989, D. Rodriguez et al., "Highly Attenuated Vaccinia Virus Mutants for the Generation of Safe Recombinant Viruses", pp. 1287-1291, see entire document.	5,14,35,36,59, 6 0 , 6 2 - 6 5 / 7 , 9 , 1 1 - 1 3 , 1 5 - 34,38,53,56,57
X/Y	WO, A, 90/12101 (Smith et al.) 18 October 1990, see entire document.	1 - 6 , 3 5 / 7 - 34,36,59-66
X/Y	Virology, Volume 152, issued 1986, M. E. Perkus et al., "Insertion and Deletion Mutants of Vaccinia Virus", pp. 285-297, see entire document.	1 - 6 , 3 5 / 7 - 34,36,59-66
X/Y	Virology, Volume 179, Number 1, issued November 1990, M. E, Perkus et al., "Vaccinia Virus Host Range Genes", pp. 276-286, see entire document.	1,2,5,7,9/11- 36,59,60,62-65
X/Y	Nature, Volume 317, issued 31 October 1985, R. M. L. Buller et al., "Decreased Virulence of Recombinant Vaccinia Virus Expression Vectors is Associated with a Thymidine Kinase-Negative Phenotype", pp. 813-815, see entire document.	1 4,6,8,10,14,35 ,36,59,60,61- 66/15-34

* Special categories of cited documents:[16]		"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	
"E"	earlier document but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	
"P"	document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

IV. CERTIFICATION	
Date of the Actual Completion of the International Search[2] 02 JUNE 1992	Date of Mailing of this International Search Report[2] 24 JUN 1992
International Searching Authority[1] ISA/US	Signature of Authorized Officer[20] *Deborah Fresa (or* D. L. BARND

Form PCT/ISA/210 (second sheet)(May 1986) &

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET		
X/Y	Journal of Virology, Volume 62, No. 12, issued December 1988, H. Shida et al., "Effects and Virulences of Recombinant Vaccinia Viruses Derived from Attenuated Strains That Express the Human T-Cell Leukemia Virus Type I Envelope Gene", pp. 4474-4480, see entire document.	1 4,6,8,10,35,59 -66/14-34,36
Y	Critical Reviews in Immunology, Volume 10, No. 1, issued 1990, J. Tartaglia et al., "Poxvirus-Based Vectors as Vaccine Candidates", pp. 13-30, see entire document.	1-66

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the international Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet(2))(Rev. 4-90) &

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document,[18] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
X/Y	WO, A, 90/10693 (Paoletti et al.) 20 September 1990, see entire document.	1 5,7,9,14,35,36,59-66/11-13,15-34
X/Y	Journal of Virology, Volume 63, No. 9, issued September 1989, M. E. Perkus et al., "Cloning and Expression of Foreign Genes in Vaccinia Virus, Using a Host Range Selection System", pp. 3829-3836, see entire document.	1-5,7,9/11-55,61,66
Y	The Lancet, Volume 337, No. 8741, issued 09 March 1991, E. L. Cooney et al., "Safety of and Immunological Response to a Recombinant Vaccinia Virus Vaccine Expressing HIV Envelope Glycoprotein", pp. 567-572, see entire document.	14,24,36,38,53,56,57,59
Y	Proceedings of the National Academy of Sciences USA, Volume 86, issued September 1989, S. Hu et al., "Protection of Macaques Against Simian AIDS by Immunization with a Recombinant Vaccinia Virus Expressing the Envelope Glycoproteins of Simian Type D Retrovirus", pp. 7213-7217, see entire document.	14,25,36,51
Y	Vaccine, Volume 6, issued December 1988, J. Taylor et al., "Fowlpox Virus as a Vector in Non-Avian Species", pp. 466-467, see entire document.	37-60,62-65
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